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INDEX

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CONTENTS OF VOLUME 53, No. 1, 1953

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PAGE

Mode of action of high-voltage cathode rays on aqueous solutions of amino-acids. By B. E. Proctor and D. S. Bhatia. (With four figures) 1

A Couette viscosimeter. By A. G. Ogston and J. E. Stanier. (With two figures) 4

Studies involving enzymic phosphorylation. 2. Changes in the hexokinase activity of the small intestine of rats caused by feeding different diets. By C. Long. (With one figure) 7

The sulphatase of ox liver. 1. The complex nature of the enzyme. By A. B. Roy. (With six figures) . 12

Studies on ali-esterases and other lipid-hydrolysing enzymes. 1. Inhibition of the esterases and acetoacetate production of liver. By D. K. Myers and B. Mendel 16

Amino-acid utilization in bacterial growth. 1. Peptide utilization by a leucine-requiring mutant of *Escherichia coli*. By Marie-Louise Hirsch and G. N. Cohen 25

Acetylcholine. 1. Hydrolysis by hydrogen and hydroxyl ion. By J. Butterworth, D. D. Eley and G. S. Stone. (With five figures) 30

Acetylcholine. 2. The heat of hydrolysis. By G. S. Annis and D. D. Eley. (With one figure) . . . 34

Observations on the composition and activity of partially arylated insulin. By G. L. Mills . . . 37

Sedimentation and diffusion studies on insulin: the maximum molecular weight. By J. M. Creeth . . 41

The metabolism of collagen from liver, bone, skin and tendon in the normal rat. By A. Neuberger and H. G. B. Slack 47

Differentiation of ribonucleic acids by inhibition of streptococcal deoxyribonuclease. By A. W. Bernheimer 53

A biological method of assay of hyaluronidase. By R. Jaques 56

Hyaluronidase: correlation between biological assay and other methods of assay. By J. H. Humphrey and R. Jaques 59

The differentiation of true and pseudo cholinesterase by organo-phosphorus compounds. By W. N. Aldridge 62

Studies on fumarase. 2. The effects of inorganic anions on fumarase activity. By V. Massey. (With six figures) 67

Studies on fumarase. 3. The effect of temperature. By V. Massey. (With thirteen figures) 72

The electrophoresis of acid mucopolysaccharides on filter paper. By K. G. Rienits. (With five figures) 79

Studies in the bile acids. 1. A method of separation and identification. By I. D. P. Wootton. (With two figures) 85

Enzyme inhibitions by snake venoms. By B. M. Braganca and J. H. Quastel. (With fifteen figures) . 88

The phytase of wheat. By F. G. Peers. (With eight figures) 102

Serum esterases. 1. Two types of esterase (A and B) hydrolysing *p*-nitrophenyl acetate, propionate and butyrate, and a method for their determination. By W. N. Aldridge. (With seven figures) . . . 110

Serum esterases. 2. An enzyme hydrolysing diethyl *p*-nitrophenyl phosphate (E 600) and its identity with the A-esterase of mammalian sera. By W. N. Aldridge. (With eight figures) 117

Studies in detoxication. 45. β-Glucuronidase and arylsulphatase in the crop fluid of locusts. By D. Robinson, J. N. Smith and R. T. Williams. (With one figure) 125

Studies in detoxication. 46. The metabolism of aliphatic alcohols. The glucuronic acid conjugation of acyclic aliphatic alcohols. By I. A. Kamil, J. N. Smith and R. T. Williams 129

PAGE

Studies in detoxication. 47. The formation of ester glucuronides of aliphatic acids during the metabolism of 2-ethylbutanol and 2-ethylhexanol. By I. A. Kamil, J. N. Smith and R. T. Williams . . 137

Studies on the metabolism of semen. 8. Ergothioneine as a normal constituent of boar seminal plasma. Purification and crystallization. Site of formation and function. By T. Mann and E. Leone. (With two figures) 140

Quantitative paper chromatography of reducing steroids of the adrenal cortex. By V. Schwarz. (With three figures) 148

Studies in rhodopsin. 6. Regeneration of rhodopsin. By F. D. Collins, J. N. Green and R. A. Morton. (With two figures) 152

Spectrophotometric determination of fructose-1:6-diphosphate, hexosemonophosphates, adenosinetriphosphate and adenosinediphosphate. By E. C. Slater. (With five figures) 157

The effect of thiol and other group-specific reagents on erythrocyte and plasma cholinesterases. By L. A. Mounter and V. P. Whittaker. (With two figures) 167

The effect of dosage on sexual differences in the iron metabolism of rats. By E. M. Widdowson and R. A. McCance. (With one figure) 173

Proceedings of the Biochemical Society

Malonate inhibition of succinic dehydrogenase. By M. B. Thorn i

The adeninenucleotide specificity of oxidative phosphorylation. By E. C. Slater and F. A. Holton . ii

Transfructosidation by mould invertase preparations. By J. Edelman and F. J. Bealing . . . ii

The conversion of naphthalene to 1:2-dihydronaphthalene-1:2-diol monoglucuronide in the rabbit. By E. D. S. Corner, F. S. Billett and L. Young iii

Stereochemical aspects of the metabolism of ethylbenzene. By J. N. Smith, R. H. Smithies and R. T. Williams iv

Glucosulphatase activity in marine molluscs. By K. S. Dodgson and B. Spencer iv

Composition and cytology of *Nitrosomonas*. By T. Hofman and H. Lees v

The chromatography of bile pigments with particular reference to the Van den Bergh reaction. By P. G. Cole and G. H. Lathe vi

The application of counter-current methods to the fractionation of lipid material from brain. By P. G. Cole, G. H. Lathe and C. R. J. Ruthven vi

A new brief method of estimating net protein value. By A. E. Bender and D. S. Miller . . . vii

Constancy of the N/H_2O ratio of the rat and its use in the determination of the net protein value. By A. E. Bender and D. S. Miller vii

The incorporation of labelled phosphate into the lipids of a guinea pig brain homogenate. By R. M. C. Dawson viii

Two selective inhibitors of cholinesterase. By L. Austin and W. K. Berry ix

INDEX OF AUTHORS

	PAGE
Aldridge, W. N.	62, 110, 117
Annis, G. S.	34
Austin, L.	ix
Bealing, F. J.	ii
Bender, A. E.	vii
Bernheimer, A. W.	53
Berry, W. K.	ix
Bhatia, D. S.	1
Billett, F. S.	iii
Braganca, B. M.	88
Butterworth, J.	30
Cohen, G. N.	25
Cole, P. G.	vi
Collins, F. D.	152
Corner, E. D. S.	iii
Creeth, J. M.	41
Dawson, R. M. C.	viii
Dodgson, K. S.	iv
Edelman, J.	ii
Eley, D. D.	30, 34
Green, J. N.	152
Hirsch, Marie-Louise	25
Hofman, T.	v
Holton, F. A.	ii
Humphrey, J. H.	59
Jaques, R.	56, 59
Kamil, I. A.	129, 137
Lathe, G. H.	vi
Lees, H.	v
Leone, E.	140
Long, C.	7
McCance, R. A.	173
Mann, T.	140
Massey, V.	67, 72
Mendel, B.	16
Miller, D. S.	vii
Mills, G. L.	37
Morton, R. A.	152
Mounter, L. A.	167
Myers, D. K.	16
Neuberger, A.	47
Ogston, A. G.	4
Peers, F. G.	102
Proctor, B. E.	1
Quastel, J. H.	88
Rienits, K. G.	79
Robinson, D.	125
Roy, A. B.	12
Ruthven, C. R. J.	vi
Schwarz, V.	148
Slack, H. G. B.	47
Slater, E. C.	157, ii
Smith, J. N.	125, 129, 137, iv
Smithies, R. H.	iv
Spencer, B.	iv
Stanier, J. E.	4
Stone, G. S.	30
Thorn, M. B.	i
Whittaker, V. P.	167
Widdowson, E. M.	173
Williams, R. T.	125, 129, 137, iv
Wootton, I. D. P.	85
Young, L.	iii

Mode of Action of High-voltage Cathode Rays on Aqueous Solutions of Amino-acids

By B. E. PROCTOR and D. S. BHATIA*
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(*Received 22 April* 1952)

Previous reports by the authors (Bhatia & Proctor, 1951; Proctor & Bhatia, 1950, 1952) have shown that irradiation of haddock fillets by cathode rays resulted in no significant loss of amino-acids in fish muscle, but that cathode-ray irradiation of aqueous solutions of histidine monohydrochloride, tryptophan, tyrosine, phenylalanine, and cystine resulted in the decomposition of these amino-acids. Evidence has been presented that the histidine molecule is deaminated and that the glyoxaline ring of histidine is broken by irradiation with cathode rays. Evolution of hydrogen sulphide upon irradiation of aqueous solutions of cystine indicated that the molecule of cystine is decomposed at the disulphide linkage.

Table 1. *Yields of ammonia from amino-acids in* 0·1 M *solution in* 0·1 N-HCl *upon irradiation with a cathode-ray dose of* 250 000 *r.e.p.*

Amino-acid	NH_3-N (μg./ml.) Replicates				Average	NH_3 (μg./ml.)
Histidine monohydrochloride*	11·24	11·22	11·22		11·23	13·63
Cystine	5·61	5·49	6·09	6·03	5·81	7·05
Phenylalanine	3·87	3·84	3·36		3·69	4·48
Tyrosine	3·33	3·27	3·48	3·18	3·32	4·03
Tryptophan	1·29	1·29	1·38		1·32	1·60

* In a previous report (Bhatia & Proctor, 1951), deamination studies were conducted on solutions containing 100 μg. histidine monohydrochloride monohydrate/ml.

Allen, Steiger, Magill & Franklin (1937) reported that several amino-acids and their derivatives undergo change when exposed to the influence of cathode rays and ultraviolet radiations. Ammonia was liberated from all compounds upon irradiation. Deamination of amino-acids upon irradiation with X-radiation has been observed also by Stein & Weiss (1948) and by Dale, Davies & Gilbert (1949).

Arnow (1942) and Spiegel-Adolf (1937) noted that phenylalanine solutions became pigmented when exposed to ultraviolet rays. Irradiated neutral, acid, or alkaline solutions of phenylalanine were found by Arnow to give a positive Millon test, indicating the formation of phenol. Tyrosine has also been investigated by Arnow (1937), who found that, in air, tyrosine was converted partly into 3:4-dihydroxyphenylalanine (DOPA). Schocken (1950) has reported on the effect of ultraviolet irradiation on certain amino-acids. The degree of conversion of phenylalanine to tyrosine and of tyrosine to DOPA has been determined.

The present investigation was conducted to elucidate the mode of chemical action of cathode rays on certain amino-acids.

Table 2. *Effect of irradiation with high-voltage cathode rays on* pH *of solutions of amino-acids*

Dose (r.e.p.)	pH of solution: Phenylalanine (200 μg./ml.)	Tryptophan (50 μg./ml.)	Tyrosine (100 μg./ml.)
Control (non-irrad.)	2·66	2·94	2·96
100 000	2·64	2·94	2·80
200 000			2·76
250 000	2·64	2·94	
400 000			2·77
500 000	2·65	2·94	
800 000			2·77
1 000 000	2·65	2·93	
1 500 000			2·85

EXPERIMENTAL AND RESULTS

The amino-acids used were L-histidine monohydrochloride monohydrate, DL-phenylalanine, DL-tryptophan, L-tyrosine, and L-cystine.

Solutions (0·1 M) of these amino-acids were prepared in 0·1 N-HCl; 3 ml. samples of the solutions were sealed into

* Present address: Central Food Technology Research Institute, Mysore, India.

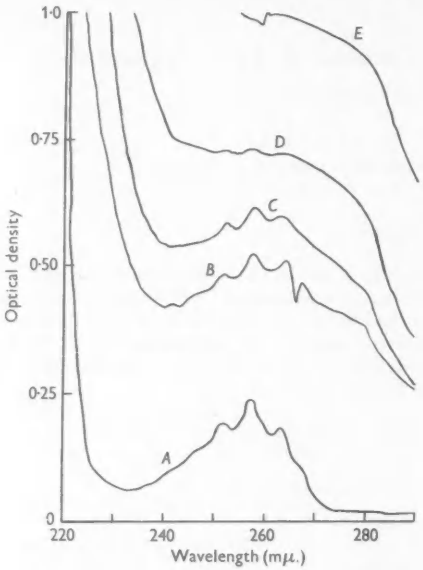

Fig. 1. Ultraviolet absorption spectra of irradiated and control solutions of DL-phenylalanine (concn. 200 μg./ml.). Cathode-ray dose: curve *A*, non-irradiated; curve *B*, 100 000 r.e.p.; curve *C*, 250 000 r.e.p.; curve *D*, 500 000 r.e.p.; curve *E*, 1 000 000 r.e.p.

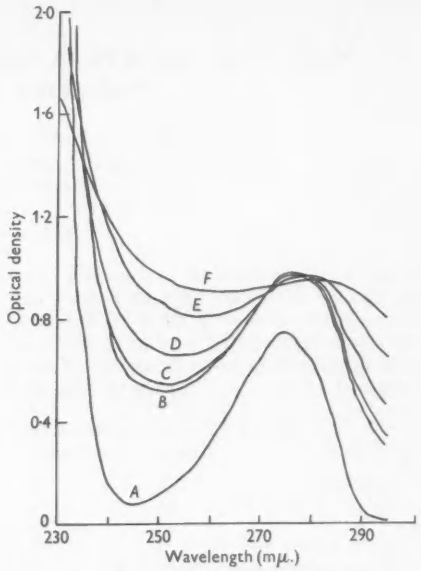

Fig. 3. Ultraviolet absorption spectra of irradiated and control solutions of L-tyrosine (concn. 100 μg./ml.). Cathode-ray dose: curve *A*, non-irradiated; curve *B*, 100 000 r.e.p.; curve *C*, 200 000 r.e.p.; curve *D*, 400 000 r.e.p.; curve *E*, 800 000 r.e.p.; curve *F*, 1 500 000 r.e.p.

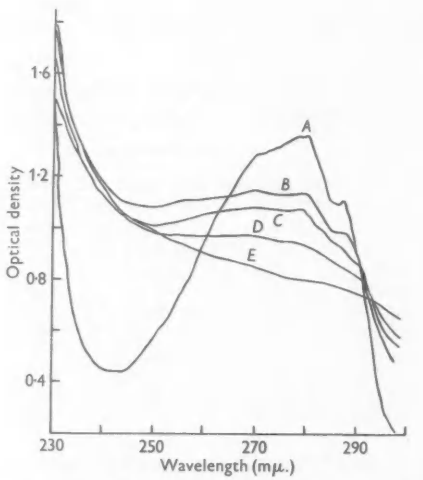

Fig. 2. Ultraviolet absorption spectra of irradiated and control solutions of DL-tryptophan (concn. 50 μg./ml.). Cathode-ray dose: curve *A*, non-irradiated; curve *B*, 100 000 r.e.p.; curve *C*, 250 000 r.e.p.; curve *D*, 500 000 r.e.p.; curve *E*, 1 000 000 r.e.p.

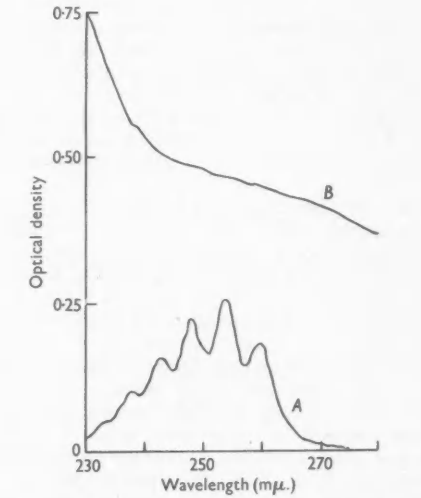

Fig. 4. Ultraviolet absorption spectra of irradiated and control solutions of benzène in water (37·2 mg./100 ml.). Cathode-ray dose: curve *A*, non-irradiated; curve *B*, 1 500 000 r.e.p.

5 ml. glass ampoules, and the ampoules were subjected to electron irradiation.

Replicate determinations of free NH_3 by the micro-diffusion method (acidimetric) as described by Conway (1947) were made on solutions of these amino-acids after exposure to a cathode-ray dose of 250 000 r.e.p. (Table 1).

Spectrophotometric determinations of the absorption spectra of three of the amino-acids were made (Figs. 1–3). For these determinations, suitable concentrations of the amino-acids were chosen, and the solutions were exposed to graded doses of cathode rays.

Determinations of pH were made on solutions of the same three amino-acids (Table 2). No significant change was noted in the pH of the solutions of phenylalanine and tryptophan upon irradiation with increasingly greater doses of cathode rays, but a slight drop was noted in the pH of solutions of tyrosine.

The techniques used were the same as described previously (Bhatia & Proctor, 1951; Proctor & Bhatia, 1952).

DISCUSSION

It has been demonstrated that amino-acids are deaminated upon irradiation with high-voltage cathode rays. As shown in Table 1, histidine was deaminated to the greatest extent and the order of deamination runs as follows: histidine > cystine > phenylalanine > tyrosine > tryptophan. As reported previously (Bhatia & Proctor, 1951), ammonia nitrogen evolved upon irradiation of histidine is contributed both by the nitrogen in the imidazole ring and the α-amino group. In their experiments with X-rays, Dale *et al.* (1949) observed the phenomenon of the specificity of the deamination of amino-acids and other nitrogen-containing compounds.

The relative radiosensitivities, based on microbiological assay data and expressed in terms of the specific inactivation dose (D/C) in r.e.p./g./ml., of these amino-acids have been calculated. They are as follows: histidine, $1{\cdot}25 \times 10^9$; phenylalanine, $1{\cdot}7 \times 10^9$; cystine, $2{\cdot}40 \times 10^9$; tyrosine, $2{\cdot}60 \times 10^9$; tryptophan, $10{\cdot}00 \times 10^9$.

In the studies on ultraviolet absorption spectra, phenylalanine showed absorption minima at 233, 254 and 261 mμ. and maxima at 252, 257 and 263 mμ. The selective absorption changed with increasing doses of radiation, and at the doses of 500 000 and 1 000 000 r.e.p. the peaks essentially disappeared, indicating that the benzene ring had been split open. There was a considerable increase in the absorption, which probably was due to the molecular fragments obtained upon irradiation of the molecule of phenylalanine.

Tryptophan was characterized by absorption minima at 242 and 286 mμ. and maxima at 280 and 287 mμ. Upon irradiation, both the maxima and the minima disappeared, which is evidence that the indole ring of tryptophan was broken.

Tyrosine was characterized by an absorption minimum at 245 mμ. and a maximum at 274–275 mμ. When tyrosine solutions were irradiated at 1 500 000 r.e.p., the peaks disappeared and a curve with a general absorption was obtained.

Irradiation of the three aromatic amino-acids, tryptophan, phenylalanine, and tyrosine has indicated that cathode rays are capable of splitting the benzene ring. To confirm this observation further, an aqueous solution of benzene was exposed to a cathode-ray dose of 1 500 000 r.e.p. and ultraviolet absorption spectra were determined with irradiated and non-irradiated solutions. Fig. 4 demonstrates clearly that the selective absorption characteristic of benzene disappears upon irradiation. This indicates beyond any doubt that the benzene ring is broken.

SUMMARY

1. Exposure of amino-acids to high-voltage cathode rays results in deamination.

2. Determinations of the ammonia yields from 0·1 M solutions of certain amino-acids upon irradiation with a cathode-ray dose of 250 000 r.e.p. indicate that the order of deamination of the amino-acids studied was: histidine > cystine > phenylalanine > tyrosine > tryptophan.

3. Spectroscopic evidence is presented that irradiation with high-voltage cathode rays causes the fission of the ring structure of the aromatic amino-acids.

Appreciation is expressed to Prof. John G. Trump and Mr Kenneth A. Wright of the Department of Electrical Engineering, Massachusetts Institute of Technology, for permission to use the electrostatic generator in their laboratory.

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A Couette Viscosimeter

By A. G. Ogston and J. E. Stanier*
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(*Received* 4 *April* 1952)

Ogston & Stanier (1950, 1951, 1952) have referred to measurements made with a Couette viscosimeter. Since this viscosimeter can be constructed almost wholly in an ordinary laboratory workshop, and since it has proved to be convenient and accurate in use, a description of its details is given here. With some modification, the same instrument has been used for measuring streaming birefringence. The basic design is that of Lawrence, Needham & Shen (1944), the main features and dimensions being copied from their design.

* With assistance in the design and construction from J. T. Cox.

Construction

Drive. A type RQ synchronous motor, 73 rev./min. (Drayton Regulator and Instrument Co. Ltd.) is coupled by a stirrup coupling to the plate of a ball-type variable gear (Variable drive Mk III Bombing trainer Mk III). The cylinder shaft of the gear carries a triple pulley, diameters 2·75, 2 and 1·25 in. This pulley drives a corresponding pulley on the viscosimeter by a loop of soft cotton string, long-spliced, and run moderately tight. Speeds of revolution of the viscosimeter pot between 0 and 150 rev./min. can thus be obtained.

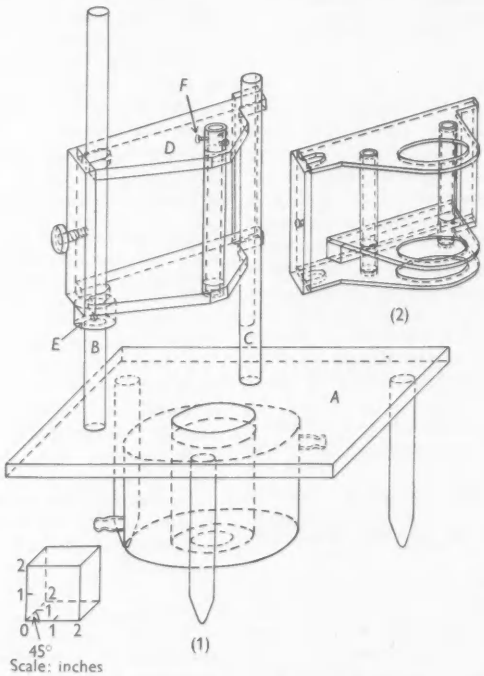

Fig. 1. Isometric (45°) sketches of (1) framework and viscosimeter head; (2) birefringence head. Certain constructional details have been omitted, including two vertical tubes on the viscosimeter head, the graduated rings on the birefringence head, and the screw feet on the framework.

Viscosimeter framework (Fig. 1 (1)). This consists of a 0·5 in. brass base-plate *A*, 12 in. square, supported on three steel legs, $\frac{7}{8}$ in. diam. and 6 in. long, two of which are fitted with screw feet for levelling. The plate carries a 0·75 in. steel rod *B*, 15 in. long, and a similar rod *C* milled flat on one side; these carry and locate the head *D*.

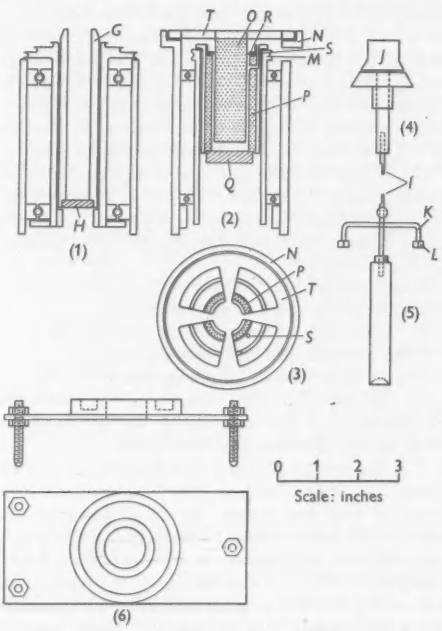

Fig. 2. (1) Section of viscosimeter bearing assembly and pot. (2) Section of birefringence bearing assembly, pot and inner cylinder, with Perspex annulus in place. (3) Plan view of (2). (4) Viscosimeter torsion head. (5) Viscosimeter bob, with damping arms and mirror. (6) Elevation and plan of viscosimeter damping trough. Transparent parts are hatched, ebonite stippled.

Below the centre of the plate is brazed a brass cylinder $2\frac{7}{8}$ in. internal diam. and 4·25 in. deep: this is open at the top and closed at the bottom, except for a central hole 1·5 in. diam. Round this cylinder is brazed a cylindrical water jacket, 6 in. diam. and 4·25 in. deep, closed except for two tubes through which to circulate water.

Bearing assembly for the rotating cylinder (Fig. 2 (1)). An outer brass cylinder $2\frac{7}{8}$ in. diam. is an easy push fit into the cylinder of the framework. Two ball-races separate this from the inner brass cylinder. This is open at both ends, of 1 in. internal diam. and 4 in. long. It carries a narrow annular flange at its lower end to support the steel viscosimeter pot *G*: its upper end carries the triple pulley mentioned above, this standing just clear of the framework base-plate.

Outer viscosimeter cylinder ('*pot*') (Fig. 2 (1) *G*). This is of stainless steel, $4\frac{3}{8}$ in. long, external diam. 1 in., internal diam. $\frac{5}{8}$ in. (1·570 cm.). It is a push fit into the bearing assembly. Difficulty was experienced in machining the bore, within the limits required (0·0005 in.), by the normal boring technique on a Smart and Brown Model '*A*' lathe, as it was found that the boring tool wandered. The pot was eventually made for us by the Pressed Steel Co., Oxford, by grinding, the inner bore being polished, uniform and coaxial within the limits stated. The open lower end is recessed to take a glass or Perspex bottom (Fig. 2 (1) *H*).

Inner viscosimeter cylinder ('*bob*') (Fig. 2 (5)). This was made on the laboratory lathe, of stainless steel, length $3\frac{1}{8}$ in., diam. 0·5 in. (1·258 cm.). Its lower end is made concave to trap an air bubble, as recommended by Lawrence *et al.* (1944) to reduce end effects: its upper end is drilled centrally $\frac{3}{32}$ in. diam., fitted with a small grub-screw, to secure the suspension. Both the steel pot and bob must be demagnetized after machining.

Viscosimeter head. (Fig. 1 *D*). This was so constructed that the suspended bob could be raised and swung out of the way and could be returned to exactly its former position. The carriage consists of two parallel horizontal plates of duralumin, $\frac{3}{8}$ in. thick and 4·5 in. apart. They are fastened together by three vertical duralumin tubes, 0·75 in. external diam., 0·5 in. internal diam., cemented with Araldite (Aircraft Accessories Ltd.). A vee, with steel inserts at one corner of each plate, slides on the circular rod *B* carried by the framework, and a cross-piece carries a locking screw. The opposite side of the carriage carries a magnet on a cross-piece, which locates against the flat-sided rod, *C*, of the framework. The vertical position of the carriage is fixed by a stop-ring, *E*, which is located on the circular rod, *B*, of the framework by a set-screw.

One of the three duralumin tubes is approximately vertically over the centre of the bearing assembly, when the carriage is located on the two rods. The suspension wire for the bob runs in this tube and is secured, by a grub-screw, into the torsion head (Fig. 2 (4)). This last is located within the top of the duralumin tube by three set-screws, *F*, set at 120°, which allow the inner cylinder to be exactly centred.

Suspension of the bob (Fig. 2 (4) and (5)). Eureka wire of 40–45 s.w.g. has been found satisfactory for this purpose, showing no fatigue or hysteresis over the range of torsions used. The length of wire used is about 7·5 in. It is soldered at each end to brass rods of $\frac{3}{32}$ in. diam.; these are filed so that the wire is central with the rods (*I*). The lower rod is 2 in. long

and fits 0·5 in. into the bob, being secured by a grub-screw. The upper rod, 1 in. long, is similarly secured to the torsion head (*J*). The lower rod has a galvanometer mirror of 1 m. radius cemented to its upper end and also carries the torsional damping device.

Torsional damping (Fig. 2 (5) and (6)). This was found greatly to increase the steadiness of the readings of deflexion. It consists of a crossbeam of aluminium wire (*K*), 2 in. long, cemented to the lower brass rod. The ends are bent downward 0·5 in. and each carries a cross-shaped paddle, *L*, of aluminium foil, 0·25 in. across and $\frac{1}{8}$ in. deep. These paddles rotate freely in an annular trough 0·5 in. broad and 0·25 in. deep, containing propylene glycol. This trough (Fig. 2 (6)) is made of Perspex and it stands on levelling screws on the framework baseplate, straddling the stroboscope. Care is required that the damping fluid does not contain dust.

Measurement of speed. At speeds less than $16\frac{2}{3}$ rev./min., revolutions are counted against a stop-watch. For higher speeds, a stroboscope disk was made by marking degrees from a circular protractor and making segments alternately black with Indian ink. A hole in the centre fits over the top of the pot. The stroboscope is observed with a neon lamp and gives speeds of $16\frac{2}{3}$, $33\frac{1}{3}$, 50, $66\frac{2}{3}$, $83\frac{1}{3}$, 100, $133\frac{1}{3}$, and 150 rev./min.

Measurement of deflexion. The galvanometer mirror is arranged to project an image of a vertical hair line on to a circular scale, at its focal distance (50 cm.). The scale is made from $\frac{1}{8} \times 1$ in. brass strip, bent into a circle of 50 cm. radius, with millimetre graph paper stuck on to it. Its useful length is 100 cm.

Control of temperature. This is effected by circulating water from a cylindrical thermostat tank. Circulation is by a Stewart Turner 120 G.P.H. centrifugal pump, which also stirs the thermostat. A Sunvic thermostat type TS 3, with a 100 W. bulb controls the temperature to better than 0·1°. The contents of the viscosimeter come to within 0·1° of the temperature of the circulating water after 15–20 min. It was, therefore, found to be unnecessary to fill the bearing space with paraffin, as Lawrence *et al.* (1944) recommend.

Adjustment and use

Levelling. It is important that the axis of rotation should be vertical. This is best achieved by levelling the pot directly, by means of a sensitive clinometer, placed on top of the pot. The framework is levelled, by means of its levelling screws, until the clinometer shows a constant reading when the pot is rotated through 360°.

Centring. It is estimated that the bob can be centred to within 0·1 mm. by eye alone, and no variations ascribable to imperfect centring have been observed. The pot is filled with water (10 ml.) and the bob is lowered until its lower end is about 1·5 cm. above the bottom of the pot; the water level is then about 1 cm. above the top of the bob. The stop ring (Fig. 1 *E*) is locked in position. The position of the bob is observed from below, by means of a mirror, and the set-screws (Fig. 1 *F*) are adjusted until the bob is central in the pot.

Assembly for use. The thermostat circulation is started. The pot is put into place and filled with 10 ml. of fluid: this has first been de-gassed to prevent the formation of bubbles. The stroboscope and damping trough are placed in position, and the bob is lowered until the carriage rests on the stop ring. The fluid is inspected from below, to see that no bubbles have been included, except in the concavity of the bob. The hair line is brought to a convenient zero. When temperature equilibrium has been established, the deflexions are noted at varying speed, a few seconds only being required to obtain a steady reading.

Range of usefulness. Water gives a scale deflexion of about 10 cm. with a 40 s.w.g. wire at 100 rev./min. (velocity gradient 50 sec.$^{-1}$), accurate to 0·2 cm. or better; at higher speeds, the bob develops a wobble. With viscous liquids speeds up to 150 rev./min. (velocity gradient 75 sec.$^{-1}$) can be used without wobbling of the bob. Steady and reproducible readings are obtained at 0·3 rev./min. or less.

Test of accuracy. The absolute viscosity of distilled water was determined at 25°. The torsional constant of the suspension (44 s.w.g. Eureka) was determined by measuring the natural frequency of the bob and its moment of inertia (14·3 g. cm.^{2}). The plot of deflexion against speed was linear, with a standard deviation of 0·6 %. The value estimated for η (25°) was $0{\cdot}90 \pm 0{\cdot}006 \times 10^{-2}$ poise, agreeing with the value $0{\cdot}8941 \times 10^{-2}$ (Landolt-Börnstein, 1927) within the standard deviation of the estimate.

Streaming birefringence

For measurement of streaming birefringence the apparatus was modified in the following ways.

Bearing assembly (Fig. 2 (2) and (3)). A separate assembly is used, with narrow ball races: inner diam. $1\frac{1}{2}$ in., outer diam. $2\frac{7}{8}$ in. The single driving pulley (*M*) has diam. $1\frac{7}{8}$ in. The outer housing carries an annular flange (*N*), to support the inner cylinder (*O*), and is cut away for the driving string to the pulley.

Outer cylinder ('*pot*') (Fig. 2 (2) and (3) *P*). This is of brass outside, and ebonite inside to reduce reflexions of light: length $2\frac{5}{8}$ in., external diam. 1·5 in., internal diam. 0·9055 in. (2·30 cm.). Its bottom is recessed to take a glass plate (*Q*) and its top to take a Perspex annulus (*R*). A flange supports it in the bearing assembly, and this flange is drilled (*S*) for filling.

Inner cylinder (Fig. 2 (2) *O* and (3)). This is of ebonite, length 2·5 in., diam. 0·7874 in. (2·00 cm.), secured to the centre of a brass disk (*T*), 0·3 in. thick, diam. 3·25 in., which rests on the flange of the bearing assembly. This disk is partly cut away, to allow viewing of the liquid. Disk and cylinder were machined together, so as to ensure their being concentric and coaxial.

Head (Fig. 1 (2)). This is similar to the viscosimeter head, but consists of three horizontal plates. The lower two are fitted with rotating rings, engraved in degrees, to hold cross-wires, polarizer or quarter-wave plate.

Optical parts. A Pointolite lamp, with a 25 cm. lens, is used to project a vertical parallel beam through a hole in the bench below the apparatus. This beam is made vertical by marking a point on the ceiling vertically above the centre of the pot by plumb-line, and adjusting until the circle of light, projected through the pot, is centred on this point. The polarizer, a 5 cm. Nicol prism, is secured below the bench. The analyser (a similar prism) is placed on the middle deck of the head. For measuring angle of isocline, cross-wires, made of thin Nylon thread, are placed on the lowest deck: for measurement of birefringence, a $\frac{1}{4}$-wave plate is placed on the lowest deck. A field lens, on the top deck, allows the whole annulus of liquid to be viewed at one time. The glass plate (*Q*) and the annulus (*R*) must be free from birefringence. The hole in the annulus is just large enough to allow it to rotate freely round the inner cylinder. It was found that the annulus can be made from Perspex, 1–2 mm. thick, by cutting very lightly with a fretsaw and finishing with sandpaper.

Filling. The pot is set up in the bearing assembly: the Perspex annulus is placed in position: the inner cylinder is inserted through the annulus. The space between pot and inner cylinder is then filled through the filling hole *S* (Fig. 2 (2)) until the liquid surface reaches and wets the under side of the annulus; by filling slowly, formation of bubbles can be avoided.

Range. Velocity gradients up to 100 sec.$^{-1}$ are attainable with the present apparatus. There is no reason why higher speeds should not be used.

SUMMARY

1. A Couette viscosimeter is described, which can be made in a laboratory workshop.

2. The accuracy of measurement is 1–2 %. The range of velocity gradient is 0–75 sec.$^{-1}$.

3. Modification for measuring streaming birefringence is described.

Mr J. T. Cox was responsible for details of design and construction. We are most grateful to Dr H. G. Kuhn, of the Clarendon Laboratory, for the loan of optical parts. This work was supported by a Research grant from the Medical Research Council.

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Studies Involving Enzymic Phosphorylation

2. CHANGES IN THE HEXOKINASE ACTIVITY OF THE SMALL INTESTINE OF RATS CAUSED BY FEEDING DIFFERENT DIETS

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Although there is no universal agreement regarding the biochemical mechanisms of intestinal absorption, many workers at the present time accept the theory of Verzár & McDougall (1936), whereby the absorption of carbohydrate and fats takes place through the formation of phosphorylated intermediates. In a recent study, Lawrie & Yudkin (1949) determined the alkaline phosphatase activities of small intestine from rats fed diets containing different amounts of carbohydrate, fat and protein; they found that phosphatase activity was significantly higher in animals fed a high-protein or high-fat diet than in those fed a high-carbohydrate (balanced or fat-free) diet. This result would not have been expected on the basis of the theory that glucose and certain other monosaccharides pass from the lumen into the mucosal cells by enzymic phosphorylation, and from the cells to the tissue

spaces draining into the portal circulation by enzymic dephosphorylation; indeed it might well have been expected that the presence in the diet of large amounts of carbohydrate and the consequent need for absorption of monosaccharides would be more likely to lead to an increased activity of both phosphorylating and dephosphorylating enzymes in the intestinal mucosa.

The hexokinase activities of intestine and of other tissues from rats maintained on normal diets have recently been determined (Long, 1951). The present study is an extension of this work and deals with the hexokinase activities of the intestinal mucosa and muscle of rats fed on diets containing different proportions of carbohydrate and other constituents. It has been found that a high-carbohydrate (fat-free) diet leads, in the rat, to a significant increase in the hexokinase activity of intestinal mucosa above the normal level; conversely, a carbohydrate-free (high-fat) diet leads to a reduction in hexokinase activity of the mucosa. These variations in carbohydrate and fat content of the diet were found not to affect significantly the hexokinase activities of rat intestinal muscle.

EXPERIMENTAL

Materials

Animals. Male hooded Lister rats have been used throughout. For Exp. 1, weanling rats, 22 days old and weighing 44–62 g., were taken from the stock colony maintained by the Physiology department of this University. For Exp. 2, young adult males, weighing 197–364 g., were either taken from stock or obtained from the Rowett Institute, Bucksburn, Aberdeenshire; the previous diet of these adult rats consisted of rat cubes (purchased from the North-Eastern Agricultural Co-operative Society Ltd.).

Diets. The diets specified by Lawrie & Yudkin (1949) for their experiments were prepared and had the composition shown in Table 1.

Table 1. *Composition of diets*

(In addition to the constituents listed, all rats received two drops of cod-liver oil each week. The casein used was 'calcium caseinate' (Glaxo); dried brewer's yeast was obtained from Pharmaco-Chemical Products Ltd. The salt mixture was prepared according to Hubbell, Mendel & Wakeman (1937).)

	Diet (g./100 g.)			
	Basal	Fat-free	High-protein	High-fat
Sucrose	55	70	15	0
Lard	15	0	0	70
Casein	15	15	70	15
Dried brewer's yeast	10	10	10	10
Salt mixture	5	5	5	5

Methods

The rats were killed by decapitation and exsanguination. The small intestine was dissected out, washed and the mucosa scraped from the muscular portion, as previously described (Long, 1951). It should be emphasized that the amount of mucosa obtained in this way may be varied over a wide range. Thus in Exp. 1, for which mucosa only was required, it amounted to an average of 35% of the moist weight of the whole intestine; in Exp. 2, where a more thorough separation was needed, the weight of mucosa averaged 65% of the moist weight of the intestine.

Determination of hexokinase activity. For intestinal mucosa, a weighed portion was homogenized by grinding in a mortar with ice-cold 0·12 M-potassium phosphate buffer, pH 7·8, containing 0·15 M-KF (5 ml./g. tissue); for intestinal muscle, the proportions were similar but the tissue was disintegrated in an all-glass homogenizer (Potter & Elvehjem, 1936). The reaction mixture contained the following components (final concentrations in brackets): glucose (0·0024 M), potassium adenosinetriphosphate (ATP) (0·005 M), $MgCl_2$ (0·005 M), KF (0·05 M), potassium phosphate buffer, pH 7·8 (0·04 M) and KCl (0·042 M). Homogenate (0·20 ml., equivalent to 33 mg. fresh tissue) was added at zero time; total volume of reaction mixture, 0·50 ml. The amount of glucose disappearing in 4 min. at 30° in air was determined by the method of Nelson (1944). Control experiments in absence of ATP were always carried out simultaneously. This test system has been found to provide optimal conditions for glucose phosphorylation (Long, 1951).

Hexokinase activity has been expressed either as μg. glucose disappearing/33 mg. moist wt./4 min., as μl. glucose/mg. dry wt./hr. ($-Q_{glucose}$) or as μl. glucose/mg. defatted dry wt./hr., where 180 μg. glucose = 22·4 μl. Dry weights were obtained by drying weighed samples overnight at 105°.

Determination of alkaline phosphatase activity. A representative portion of mucosa (about 0·5 g.) was ground in a mortar with 10 ml. distilled water. A 1 ml. sample of this suspension was taken for dry weight determination; another portion was diluted tenfold with water and used for determining alkaline phosphatase activity. The reaction mixture contained the following components (final concentrations in brackets): disodium β-glycerophosphate (0·05 M), $MgCl_2$ (0·01 M) and veronal-carbonate buffer (King & Delory, 1940), pH 9·2 (0·0125 M). At zero time the diluted enzyme suspension (2 ml.) was added and the mixture incubated at 30° for 15 min.; total volume of reaction mixture, 7·0 ml. The reaction was stopped by addition of 3 ml. 10% (w/v) trichloroacetic acid. Duplicate 4 ml. samples of filtrate were taken for determination of inorganic phosphate according to the method of Fiske & Subbarow (1925). Zero time controls and phosphate standards were always run simultaneously. This is essentially the procedure of Motzok & Wynne (1950) (see also Motzok, 1950).

Alkaline phosphatase activity has been expressed as μl. inorganic P liberated/mg. dry wt./hr. ($Q_{phosphate}$), where 31 μg. inorganic P = 22·4 μl.

Determination of fat content of tissue. Probably no method of determining the fat content of a tissue is completely free from criticism. The following procedure has been used. To 1 g. of fresh intestinal mucosa or muscle in a Pyrex boiling tube (150 × 25 mm.) were added 10 ml. $CHCl_3$ and 5 ml. methanol, and the mixture gently heated for 2–3 hr. on a water bath (bath temp. 55–60°). After cooling, the extract was filtered and the residue re-extracted in the same way. The combined filtrates were evaporated to dryness. The dry material was then extracted three times with a mixture of 2 ml. ethanol and 1 ml. diethyl ether, with gentle warming,

the extracts being filtered each time into a tared test tube. (The residue from this extraction was completely water-soluble.) The combined filtrates were evaporated to dryness and weighed.

RESULTS

The hexokinase and alkaline phosphatase activities of small intestinal mucosa of rats fed for 8 months from weaning on basal, fat-free, high-protein and high-fat diets (Exp. 1)

Six litters, each containing four male rats, were taken at weaning and divided into four groups, each group containing one animal from each litter, so as to form a randomized block design. The six rats in each particular group were housed together in a large cage and fed the appropriate diet *ad lib.* The amounts of the various diets consumed were not recorded. Body weights, determined at weekly intervals, are illustrated graphically in Fig. 1. After about 4 months, rats on the fat-free diet, having attained an average body weight of 320 g., began to lose weight and were thereafter given one drop of linseed oil each week, for it seemed possible that these animals might be suffering from a deficiency of essential fatty acids. However, after a further 2 months of relatively constant body weight, a further weight loss was noted. It is probable that lack of essential fatty acids was not solely responsible for the loss of weight, for the high-protein diet was also completely lacking in fat and the rats in this group continued to grow throughout the experiment. Rats on the basal diet grew very well and their growth was not inferior to that of stock animals on a diet of rat cubes.

Three of the animals on the fat-free diet and one on the basal diet died after about 7 months. After 8 months the remaining twenty animals were killed and determinations of hexokinase and alkaline phosphatase activities were made on the intestinal mucosa. The results obtained are shown in Table 2.

It should be mentioned here that when the hexokinase activity determinations were being planned, it was intended to assume a dry weight equal to 20·4 % of the fresh moist weight, the value found for the intestinal mucosa of rats on a stock diet (Long, 1951). For that reason, individual dry-weight determinations were not made. After the experiments were concluded, however, it did not seem justifiable to use this fixed value for percentage dry weight, in view of the widely different amounts of fat in the diet of the experimental animals. The results in Table 2 were therefore calculated in terms of fresh weight of tissue. If the dry weight of the intestinal mucosa of rats on the basal diet is assumed to be 20·4 % of the moist weight, as for animals on the stock diet, then the value for $-Q_{glucose}$ may be calculated to be 18·0 ± 1·3, which is in reasonable agreement with the

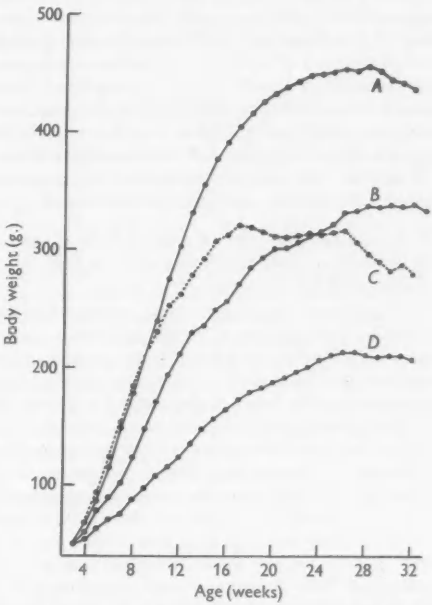

Fig. 1. Growth of weanling rats. Diets: *A*, basal; *B*, high-protein; *C*, fat-free; *D*, high-fat. Composition of diets shown in Table 1. Each point represents the mean weight of five or six rats (Exp. 1).

Table 2. *Effect of diet on hexokinase and alkaline phosphatase activity of rat intestinal mucosa (Exp. 1)*

(Weanling rats fed experimental diets for 8 months. Composition of diets given in Table 1. Values quoted are mean ± standard error of mean. Hexokinase activity expressed as μg. glucose disappearing/33 mg. moist weight/4 min.; alkaline phosphatase activity expressed as $-Q_{phosphate}$ (μl. P/mg. dry wt./hr.).)

	Diet			
	Basal	Fat-free	High-protein	High-fat
No. of animals	5	3	6	6
Body wt. (g.)	414 ± 22	283 ± 34	330 ± 7	204 ± 20
Moist wt. of whole small intestine (g.)	12·0 ± 0·9	8·1 ± 1·4	8·6 ± 0·3	7·5 ± 0·5
Moist wt. of intestinal mucosa (g.)	3·82 ± 0·46	1·93 ± 0·55	3·62 ± 0·17	3·00 ± 0·23
Hexokinase activity	65·1 ± 4·7	79·4 ± 9·7	61·3 ± 3·0	50·4 ± 2·6
Alkaline phosphatase activity	334 ± 38	323 ± 56	324 ± 43	266 ± 32

mean value of 14·8 (range 10·1–20·2) found for stock rats (Long, 1951).

The data in Table 2 have been examined statistically. It has been found, in regard to hexokinase activity, that significant differences exist between the four diets ($P < 0.001$). The fat-free diet had the largest effect, giving a result significantly higher than the basal diet ($P < 0.05$), and the high-protein or high-fat diets ($P < 0.001$). Hexokinase activities of intestinal mucosa of rats on the basal and high-protein diets were not significantly different, but both were significantly higher than for rats on the high-fat diet ($P < 0.01$ and $P < 0.05$, respectively).

Variation in alkaline phosphatase activity between the various diets was not significant.

The hexokinase activities of small-intestinal mucosa and muscle of adult rats fed for short periods on fat-free or high-fat diets (Exp. 2)

The previous experiment has shown that the nature of the diet has a significant effect on the hexokinase activity of rat intestinal mucosa. However, it might be argued that if the results had been expressed on the basis of dry weight (as were the alkaline phosphatase activities) or defatted dry weight, these effects might not have been apparent. A second experiment was therefore carried out in which the dry weight and fat content of the mucosa were determined on samples similar to those used for hexokinase activity determinations; the muscular portion of the small intestine was also investigated. This experiment was limited to two of the diets previously used, namely the high-fat and fat-free diets, for these were already known (Exp. 1) to exhibit the most marked differences.

Ten young adult male rats were divided into two groups on the basis of initial body weight (mean value, 289 g.) and fed either of the selected diets. Animals on the high-fat diet gained an average of 21 and 50 g. after 7 and 14 days, respectively; corresponding average figures for rats fed the fat-free diet were 30 and 44 g. The rats were killed after either 7 or 14 days and determinations of hexokinase activity, dry weight and fat content were carried out on intestinal mucosa and muscle. The data obtained are shown in Table 3. Since the results obtained at the end of 7 and 14 days were not significantly different, they have been combined.

The figures in Table 3 have been examined statistically. In this way it has been deduced that the hexokinase activity of intestinal mucosa is significantly higher ($P < 0.05$) for rats on a fat-free diet than for those on a high-fat diet, independently of whether the activities are expressed in terms of moist weight, dry weight or defatted dry weight of tissue. This result is of considerable interest for it means that the different levels of hexokinase activity in rat intestinal mucosa produced by feeding the fat-free or high-fat diet over a period of 8 months from weaning (Table 2) can be obtained just as readily in adult rats after feeding for 1 or 2 weeks (Table 3). The comparable values in Tables 2 and 3 for hexokinase activities based on moist weight show very close agreement.

In contrast with the result obtained for intestinal mucosa, there were no significant differences between the hexokinase activities of intestinal muscle from rats on high-fat or fat-free diets, independently of the method of expressing the results. The $-Q_{glucose}$ values (μl. glucose/mg. dry wt./hr.) of $10·3 \pm 2·0$ and $7·6 \pm 1·5$ agree satisfactorily with the average figure of 11·8 (range 9·7–13·9) found for rats on the stock diet (Long, 1951).

DISCUSSION

Although an exact comparison cannot be made, two of the results presented in this paper seem to be at variance with the findings of Lawrie & Yudkin (1949). First, the growth rates of weanling rats on the various diets are completely different in the two investigations. Those obtained in the present work depended markedly upon the composition of the diet, rats in the basal group attaining a maximum weight of about 450 g., while those in the high-fat group reached only half this value. Lawrie & Yudkin (1949), on the other hand, found their male

Table 3. *Hexokinase activities of small-intestinal mucosa and muscle of rats fed a high-fat or fat-free diet for 7 or 14 days (Exp. 2)*

(Five young adult male rats in each group, paired according to initial body weight. Composition of diets given in Table 1. Values quoted are mean ± standard error of mean.)

	Mucosa		Muscle	
	Fat-free diet	High-fat diet	Fat-free diet	High-fat diet
Moist wt. of tissue (g.)	5·34 ± 0·23	6·50 ± 0·50	3·55 ± 0·13	3·34 ± 0·02
Dry wt. (% wet wt.)	20·8 ± 1·3	24·7 ± 2·8	20·9 ± 1·2	21·0 ± 0·8
Fat content (% wet wt.)	9·5 ± 0·9	10·6 ± 0·4	4·0 ± 0·4	4·4 ± 0·4
Hexokinase activity expressed as:				
a, μg. glucose/33 mg. moist wt./4 min.	71·9 ± 9·1	45·1 ± 2·4	37·3 ± 6·1	27·7 ± 5·6
b, μl. glucose/mg. dry wt./hr.	20·1 ± 3·4	10·8 ± 1·2	10·3 ± 2·0	7·6 ± 1·5
c, μl. glucose/mg. defatted dry wt./hr.	38·2 ± 6·8	20·2 ± 3·3	12·8 ± 2·3	9·5 ± 2·1

rats to attain a maximum weight of only 202 g., with no significant variation between the groups on different diets. It does not seem possible to attribute this lack of agreement to the fact that different strains of rats were employed, for males of the Hartwell strain used by Lawrie & Yudkin (1949) are well known to attain a body weight of about 420 g. at 6 months when fed an adequate diet (Hartwell, 1926) and are thus quite comparable with the hooded Lister rats used in the present work. One explanation of this discrepancy might be that Lawrie & Yudkin (1949), when preparing their diets, used rather highly purified materials deficient in certain essentials, so that even rats on the basal (balanced) diet grew no faster than those on the high-fat diet. On general grounds, rats on a balanced diet would be expected to grow more rapidly than those on a 70% fat diet, for animals in the latter group would satisfy their caloric needs without receiving enough protein and perhaps other nutrients for maximal rate of growth. A difference in growth rate similar to that found in the present work has also been noted by Lutwak-Mann (1952).

The values recorded for the alkaline phosphatase activities of intestinal mucosa (Table 2) show no significant differences due to variation of diet. This result also differs from that claimed by Lawrie & Yudkin (1949), who found the alkaline phosphatase activity of whole intestine to be significantly higher in rats fed a high-protein or high-fat diet than in those fed the basal or fat-free diets. These different observations, of course, are not necessarily inconsistent for they refer to mucosa and whole intestine respectively. In addition, there were considerable differences in the methods used for estimating enzymic activity and finally the effect of strain difference of rats cannot be excluded here.

The main results reported in this paper establish a clear dependence of hexokinase activity of rat intestinal mucosa upon the composition of the diet. It is noteworthy that the very significant difference between the hexokinase activities of intestinal mucosa of rats fed for 8 months from weaning on high-fat and fat-free diets could be almost exactly duplicated in experiments with adult animals after only 1 or 2 weeks on these diets (Tables 2 and 3). The differences cannot be explained on the basis of variation of dry weight or defatted dry weight of the mucosae and appears to be equally real no matter what the basis for comparison. An interesting result from Exp. 1 is the finding that while there is a general positive correlation between the proportion of carbohydrate in the diet and the hexokinase activity, this correlation is by no means absolute for there was no significant difference between the hexokinase activities of intestinal mucosa of rats on the basal and high-protein diets, although the carbohydrate contents were 55 and 15% respectively.

In an earlier paper (Long, 1951), it was calculated that the hexokinase activity of rat intestinal mucosa could account for only about 30% of the experimentally determined rate of glucose absorption. However, this result did not exclude a less important role for hexokinase in the absorptive process. If a relationship does indeed exist between rate of absorption and the activity of phosphorylating enzymes in the intestine, then the present work may recall the observations of Westenbrink (1934), who found that the rate of intestinal absorption of individual monosaccharides was considerably increased by prior feeding of the appropriate monosaccharide, even for only a few days. Further, the results obtained here might also be taken to support the theory that absorption is related solely to the enzymic activity of the mucosa, for no parallel changes in the hexokinase activity of intestinal muscle have been observed.

SUMMARY

1. The hexokinase activities of intestinal-mucosa homogenates from rats fed for 8 months from weaning on different diets have been determined. Highest activity was found in rats fed a fat-free diet and lowest in those fed a high-fat diet. Intermediate values were obtained in rats fed basal (balanced) and high-protein diets. Alkaline phosphatase activities of intestinal mucosa showed no significant differences.

2. Rats fed for only 1 or 2 weeks on high-fat and fat-free diets also showed statistical differences in the hexokinase activities of their intestinal mucosae. No differences were found between hexokinase activities of intestinal muscle of the two groups.

The author wishes to thank Messrs M. H. Quenouille and F. H. C. Marriott for statistical evaluation of the results, Miss Iris Creighton for help with the rats and Mr W. R. Ferrier for technical assistance. It is also desired to thank the Medical Research Council for an Apparatus Grant.

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The Sulphatase of Ox Liver

1. THE COMPLEX NATURE OF THE ENZYME

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The enzyme sulphatase present in many animal tissues (Fromageot, 1938) has up to the present been little studied in comparison with the corresponding enzyme of *Aspergillus oryzae*. In view of the importance of sulphuric acid esters in many metabolic processes in the animal body it appeared that the sulphatase of animal tissues might be worthy of a detailed study. The method of assay used in the present work was based on that of Robinson, Smith & Williams (1951) which utilizes potassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) as substrate, and follows the sulphatase activity by the colorimetric estimation of the liberated 4-nitrocatechol. This method is not applicable to whole homogenates of animal tissues, especially liver, as such homogenates are apparently capable of metabolizing nitrocatechol, a fact which has recently been noted by Dodgson, Spencer & Thomas (1952). The method is, however, suitable for the assay of sulphatase in the partly purified preparations used in the present investigations, as under such conditions the recovery of added nitrocatechol is quantitative.

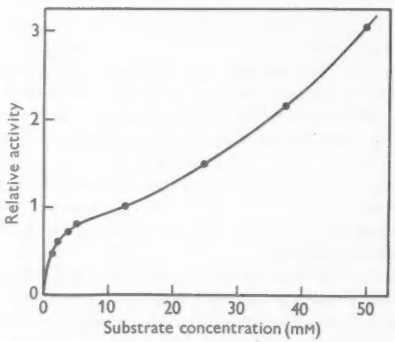

Fig. 1. Effect of varying substrate concentrations on reaction velocity. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. unfractionated sulphatase preparation. Incubated 1 hr. at 37° in 0·05 M-citrate buffer, pH 6·0.

Preliminary investigations using this substrate showed that crude sulphatase preparations gave a very anomalous substrate concentration-reaction velocity curve (Fig. 1) and it appeared that more than one enzyme might be present. This paper describes the preparation of two distinct sulphatase fractions from such crude solutions.

METHODS

Preparation of substrate

The method used was essentially that of Smith (1951), except that it was found more convenient to prepare the substrate as the dipotassium 2-hydroxy-5-nitrophenyl sulphate instead of the monopotassium salt.

To 70 g. KOH and 70 g. potassium persulphate dissolved in 1 l. water were added 30 g. *p*-nitrophenol. The reaction mixture was left 48 hr. at 37°, then acidified to pH 4 with H_2SO_4, and free phenols extracted with ether. The aqueous solution was then made strongly alkaline to litmus with KOH and concentrated *in vacuo* to about 300 ml. The solution, and any precipitate, was poured into 2 vol. acetone and the mixture filtered. After washing the residue with acetone-water (2:1, v/v), the combined filtrates were taken to dryness *in vacuo* and the residue recrystallized three times from water. Bright yellow crystals of dipotassium 2-hydroxy-5-nitrophenyl sulphate dihydrate were obtained with a yield

of about 3 g. (Found: C, 20·2; H, 1·87; N, 4·02; K, 23·0. Calc. for $C_6H_3O_7NSK_2.2H_2O$: C, 20·3; H, 2·03; N, 4·03; K, 22·5%.)

Estimation of 4-nitrocatechol

Robinson *et al.* (1951) estimated the 4-nitrocatechol by means of the red colour developed in strongly alkaline solution. This colour, although stable when pure nitrocatechol solutions were used, faded rapidly in the presence of liver homogenates, even after precipitation of the proteins. It was found by the author that this fading could be prevented and the colour stabilized by the addition of quinol, Na_2SO_3 also being added in order to prevent the rapid oxidation of the quinol in the strongly alkaline solution.

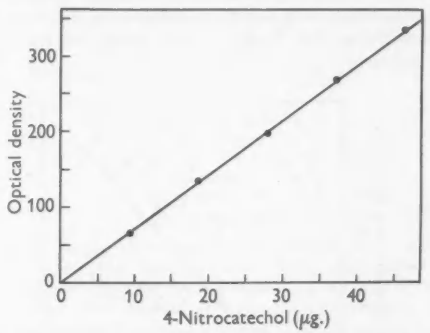

Fig. 2. Calibration curve of 4-nitrocatechol estimation.

The alkaline quinol reagent was made up immediately before use by adding 5 ml. of a 4% solution of quinol in 0·1 N-HCl (made up weekly) to 100 ml. of 2·5 N-NaOH containing 5% $Na_2SO_3.7H_2O$, giving a final quinol concentration of approximately 0·2%. The calibration curve (Fig. 2) was prepared by adding 0·2 ml. water to 0·6 ml. of standard 4-nitrocatechol solutions, followed by 3 ml. 2% phosphotungstic acid in 0·1 N-HCl. After mixing, 3 ml. of the solution were pipetted into 5 ml. freshly prepared alkaline quinol reagent. The red colours were read in a Spekker absorptiometer, using Ilford filter no. 604 (520 mμ.), against a reagent blank. The colour is stable for at least 3 hr., and the Lambert-Beer law is obeyed to an optical density of 0·7, corresponding to 100 μg. of nitrocatechol.

The intensity of the colour is independent of the concentration of the NaOH used within wide limits (1–5 N-NaOH) and of the presence or absence of Na_2SO_3. Quinol increases the intensity of the colour appreciably, but again the intensity is independent of the quinol concentration, within the limits of 0·1–0·3% in the alkaline reagent. When the phosphotungstic acid is pipetted into the alkaline quinol reagent a blue colour is produced, which fades rapidly and does not interfere with the estimations.

Preparation of the enzyme

Fresh ox liver was cut into 1 in. cubes and 100 g. portions were thrown into 500 ml. acetone at 0° in a chilled 'Atomix' blender: the mixture was homogenized for 1 min., filtered, and washed successively with 500 ml. portions of acetone and ether at 5°. After sucking dry at the pump the filter cake was broken up and dried *in vacuo* over P_2O_5. The material was sufficiently dry to powder and sieve within a few hours. When kept *in vacuo* at room temperature, the powder retained its enzymic activity for many weeks.

The enzyme solution was prepared by extracting 3 g. of the acetone powder with 20 ml. water for 1 hr. at 37°. The bulk of the debris was removed by centrifuging for 15 min. at 2000 rev./min. and the supernatant clarified by a further 30 min. centrifuging at 10 000 rev./min.

Fractionation of the enzyme

To 20 ml. of the aqueous extract prepared as above were added 2 ml. 0·1 M-phosphate buffer, pH 6·9; the mixture was cooled to 0°, and 16·5 ml. chilled acetone were slowly run in with stirring, the temperature being lowered to −9° during the process, giving a final acetone concentration of 43%, v/v. After standing 30 min. at −9°, the precipitate was removed by centrifuging at the same temperature and dissolved in 15 ml. water, giving a solution of fraction *B*. To the supernatant from this first precipitation (29 ml.), kept at −9°, was added 0·8 ml. of the phosphate buffer followed by 12·5 ml. chilled acetone, to give a final acetone concentration of 60%. The precipitate was removed by centrifuging as before and dissolved in 15 ml. water, giving fraction *A*.

Both fractions were dialysed overnight at room temperature against running water, and any insoluble material was removed by centrifuging. The solutions so obtained were diluted 5 times with distilled water to give enzyme concentrations suitable for assay by the method described below.

Estimation of enzymic activity

To 0·2 ml. 0·5 M-acetate buffer of the appropriate pH (4·7 and 5·7 in the case of fractions *A* and *B* respectively) was added 0·4 ml. of a solution of the substrate (0·006 and 0·066 M-nitrocatechol sulphate respectively for fractions *A* and *B*) adjusted to the same pH as the buffer with 0·1 N-HCl. The tubes were brought to 37°, and 0·2 ml. enzyme solution, also at 37°, was added. After incubating for 1 hr., 3 ml. 2% phosphotungstic acid in 0·1 N-HCl were added and, after mixing, the precipitated proteins removed by centrifuging. For colour development, 3 ml. of the clear supernatant were pipetted into 5 ml. alkaline quinol reagent as described above.

Assays were run in duplicate, and suitable blanks were obtained by incubating the buffered substrate in the absence of the enzyme, which was only added to the tubes immediately before precipitating the proteins with the phosphotungstic acid. The assays were read in the Spekker against the appropriate blanks.

In typical assays the amount of substrate hydrolysed during incubation was approximately 10 and 1% of that initially present in the case of fractions *A* and *B* respectively.

RESULTS

Effect of pH. The effect of pH was determined over the range 4–7 in 0·5 M-acetate buffers at a final substrate concentration of 0·003 M in the case of fraction *A* and 0·033 M in the case of fraction *B*. The substrate solutions were adjusted to pH values corresponding to those of the buffers used, as nitrocatechol sulphate is itself a strong buffer over the

pH range 5–7. This fact allowed the use of acetate buffers at the extreme alkaline end of their normal range. For the determination of the optimum pH, 0·2 ml. enzyme was added to 0·4 ml. substrate solution and 0·2 ml. 0·5M-acetate buffer, giving a final acetate concentration of 0·15M. Incubation and colour development were carried out as described above.

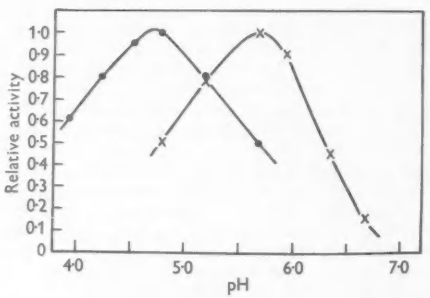

Fig. 3. Effect of pH on reaction velocity. Incubated for 1 hr. at 37° in 0·15M-acetate buffers of varying pH. Substrate concentrations 0·003 and 0·033M-nitrocatechol sulphate for fractions *A* and *B* respectively. Final volume 0·8 ml. containing 0·2 ml. enzyme solution. 'Relative activity 1·0' corresponds to a liberation of 39 and 56 μg. nitrocatechol per tube in the case of fractions *A* and *B* respectively. —●—●—, fraction *A*; —×—×—, fraction *B*.

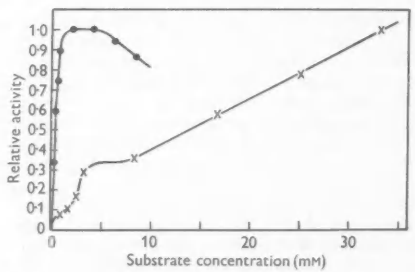

Fig. 4. Effect of varying substrate concentration on reaction velocity. Incubated for 1 hr. at 37° with varying concentrations of nitrocatechol sulphate in 0·15M-acetate buffers of pH 4·7 and 5·7 for fractions *A* and *B* respectively. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution. 'Relative activity 1·0' corresponds to a liberation of 31 and 65 μg. nitrocatechol per tube in the case of fractions *A* and *B* respectively. —●—●—, fraction *A*; —×—×—, fraction *B*.

The results are shown in Fig. 3, which indicates that the pH optima for fractions *A* and *B* are respectively 4·7 and 5·7 in 0·15M-acetate buffer, with nitrocatechol sulphate as substrate.

Effect of substrate concentration. Fig. 4 shows the effect of varying substrate concentration on the reaction velocity at a constant pH, i.e. 4·7 and 5·7 in the case of fractions *A* and *B* respectively.

With the fraction *A* there was an optimum substrate concentration of approximately 0·003M-nitrocatechol sulphate, above which concentration substrate inhibition occurred to some extent. With the *B* fraction there was no obvious substrate optimum within the limits studied. These limits were set by the solubility of the substrate and could only be overcome by radical changes in the method of assay. The hump in the curve for the *B* fraction at a concentration of 0·004M-nitrocatechol sulphate is presumably due to slight contamination by the *A* fraction.

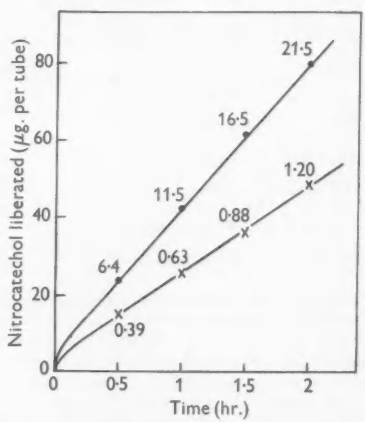

Fig. 5. Effect of time of incubation on degree of hydrolysis. In both cases the final volume of the reaction mixture was 0·8 ml. containing 0·2 ml. enzyme solution and the incubation carried out at 37°. For the assay of fraction *A* the substrate concentration was 0·003M and the buffer 0·15M-acetate, pH 4·7; for fraction *B* the corresponding figures were 0·033M and pH 5·7. The figure above each point represents the percentage hydrolysis of the substrate at the corresponding time. —●—●—, fraction *A*; —×—×—, fraction *B*.

Effect of time of incubation. Fig. 5 shows the effect of time of incubation on the degree of hydrolysis of the substrate, the enzymes working under optimal conditions of pH and substrate concentration. The degree of hydrolysis is essentially proportional to the time of incubation, apart from a slight decrease in the reaction velocity during the first 10 min. of incubation.

Effect of enzyme concentration. Fig. 6 shows the effect of varying enzyme concentrations on the reaction velocity under optimal conditions of pH and substrate concentration. With fraction *B*

there is a direct proportionality between the enzyme concentration and the reaction velocity; such a simple relationship does not appear to exist in the case of fraction *A*. This anomalous behaviour may well be due to the fraction being impure, although several preparations gave similar curves.

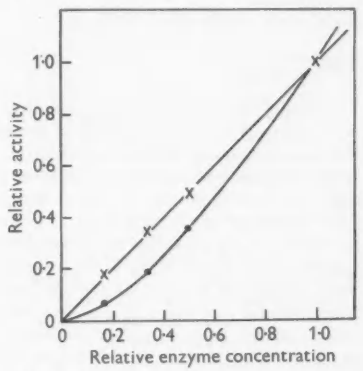

Fig. 6. Effect of enzyme concentrations on reaction velocity. Conditions as in legend of Fig. 5 except that the enzyme concentration was varied and the time of incubation kept constant at 1 hr. 'Relative velocity 1·0' represents a liberation of 75 and 51 μg. nitrocatechol per tube for fractions *A* and *B* respectively —●—●—, fraction *A*; —×—×—, fraction *B*.

Intracellular location of the enzyme. Preliminary data indicated that a considerable proportion of the sulphatase activity in a water homogenate of ox liver was insoluble, and a few experiments have been carried out with mouse liver in order to determine the localization of the sulphatase in the liver cell. These assays were carried out in 0·1 M-citrate buffer, pH 6, and in 0·05 M-nitrocatechol sulphate, thus determining essentially the activity of the *B* fraction. Cell fractionation was carried out in 0·25 M-sucrose, according to Schneider & Hogeboom (1950) and sulphatase estimations were carried out on the washed nuclear and mitochondrial fractions (sedimented at 700 and 5000 *g*. respectively) and on the supernatant from the above two fractions. Under the above conditions it was found that some 70 % of the sulphatase activity was present in the mitochondria, the bulk of the remainder being present in the supernatant fraction. These figures must be regarded as semi-quantitative due, as pointed out above, to the destruction of nitrocatechol by tissue homogenates: they indicate, however, that a high proportion of the sulphatase activity is localized in the mitochondria.

DISCUSSION

The results reported above indicate that the sulphatase of ox liver is a more complex enzyme than has previously been thought, two distinct sulphatase fractions being present. The two fractions, so far only obtained in an impure state, differ in their pH optima and in their response to variations in substrate concentration; fraction *A* having a pH optimum of 4·7 in acetate buffer and an optimum substrate concentration of 0·003 M-nitrocatechol sulphate, whereas fraction *B* has a pH optimum of 5·7 in acetate buffer and an anomalous response to variations in substrate concentration. From the data presented it may be concluded that the sulphatase of animal tissues is very different in properties from the sulphatase of *A. oryzae* which has, according to Robinson *et al.* (1951), an optimum pH of 5·9 in acetate buffer, and an optimum substrate concentration 0·0025 M-nitrocatechol sulphate. This difference in properties has not been stressed by previous workers, many of whom (Huggins & Smith, 1947; Robinson *et al.* 1951) have applied methods based on kinetic data obtained with mould sulphatase to the assay of the corresponding enzyme in animal tissues. Much of the earlier work on the animal sulphatases is also vitiated by the lack of appreciation of the insoluble nature of the enzyme in water homogenates, part of the enzyme at least being associated with the mitochondria.

SUMMARY

1. A method is described for the assay of sulphatase, using nitrocatechol sulphate as substrate.

2. Two fractions exhibiting sulphatase activity have been obtained from an aqueous extract of an acetone powder of ox liver by fractional precipitation with acetone. The properties of these fractions are given.

3. A large part of the sulphatase activity is localized in the mitochondria, and is insoluble in water homogenates.

The author wishes to express his indebtedness to Prof. R. T. Williams who supplied details of his nitrocatechol method prior to its publication. Thanks are also due to Dr J. W. Minnis who carried out the micro-analysis.

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Studies on Ali-esterases and Other Lipid-hydrolysing Enzymes

1. INHIBITION OF THE ESTERASES AND ACETOACETATE PRODUCTION OF LIVER

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Mammalian liver contains several types of esterase which might be concerned with the hydrolysis of liver lipid. The best known of these esterases are ali-esterase, lipase (Loevenhart, 1906; Willstätter & Memmen, 1924; Sobotka & Glick, 1934), lecithinase or phospholipase (King, 1931; Belfanti, Contardi & Ercoli, 1936) and cholesterol esterase (Kondo, 1910; Klein, 1938). In the present investigation interest was initially centred on the ali-esterases, that is, those esterases which act preferentially on simple aliphatic esters and triglycerides such as ethyl butyrate and tributyrin (Richter & Croft, 1942).

A fatty acid ester may be hydrolysed by several different esterases, and generally a complex mixture of esterases is present in animal tissues. Consequently it has been difficult to determine the range of specificity of the various ali-esterases. Also, the differences between ali-esterase and lipase have not been clearly defined; frequently it is assumed that the high ali-esterase activity and low lipase activity of liver are due to one and the same enzyme (cf. reviews by Bloor, 1943; Longenecker, 1946; Ammon & Jaarma, 1950).

The physiological function of lipase, lecithinase and cholesterol esterase is probably the hydrolysis and/or synthesis of substrates similar to those upon which these enzymes act most readily *in vitro*. However, the primary functional role of the ali-esterases and their physiological substrates is still largely a matter of conjecture (cf. Baker & King, 1935). Although the action of ali-esterases upon simple fatty acid esters does suggest that these enzymes are important in some stage of fat metabolism, their precise role remains problematical. Insight into their function in cellular metabolism could be obtained by inhibiting these enzymes, but the lack of a potent inhibitor of ali-esterases has previously hampered such investigations. Recently, however, it has been found that certain alkyl phosphate derivatives, commonly used as irreversible cholinesterase inhibitors, can also be employed as *in vitro* inhibitors of ali-esterase activity (Webb, 1948). This has made it possible to study the effects of ali-esterase inhibition on metabolic processes *in vitro*.

Liver tissue was used in the present investigation for the following reasons: (1) the liver is considered to be of primary importance in lipid metabolism; (2) liver lends itself readily to a study of the effects of ali-esterase inhibition on fat metabolism since fatty acid oxidation in liver cells results in an accumulation of free acetoacetate, the amount of which can easily be determined; (3) the liver is characterized by a high content of ali-esterases and virtual absence of lipase; and, if the ali-esterases play some significant role in fat metabolism, this should be especially evident in experiments with liver tissue; (4) rat liver in particular was chosen because its ali-esterase activity can be inhibited *in vivo* by administration of tri-*o*-cresyl phosphate without causing any inhibition of the true cholinesterase as it does in most other animal species (Mendel & Mortimer, 1944; cf. Mendel & Rudney, 1944). Thus in rats it is possible to compare the effects of *in vitro* and *in vivo* ali-esterase inhibition upon liver metabolism.

During the course of their studies on fatty acid oxidation in liver, Jowett & Quastel (1935*b*) investigated the relation of the ali-esterases to the spontaneous acetoacetate production of liver slices. Using atoxyl as inhibitor of the ali-esterase activity towards tributyrin (Rona & Bach, 1920), they found that the ali-esterase activity and the spontaneous acetoacetate production of rat-liver slices were inhibited to the same extent, while the oxidation of exogenous free fatty acids to acetoacetate was not greatly affected. From these data, they concluded that atoxyl inhibits the oxidation of the lipids in liver slices by depressing the 'lipase activity'.

Jowett & Quastel's results seemed to offer a satisfactory explanation of the physiological function of the ali-esterases in liver, and they were readily confirmed in the present investigation, when atoxyl was used as ali-esterase inhibitor. When, however, the more potent alkyl phosphate derivatives were used, no appreciable effect on the spontaneous acetoacetate production of liver slices could be observed in spite of the almost complete inhibition of the ali-esterase activity. This observation was surprising in view of Jowett & Quastel's results, and only after considerable investigation did we feel justified in concluding that the ali-esterases are not essential for lipid catabolism in liver slices. Our experiments

further showed that the phospholipase, lipase and ali-esterase activities of rat-liver homogenate are probably due to separate enzymes. Of these three types of esterase, only the phospholipase seems to be directly essential for lipid catabolism in liver.

METHODS

Animals and diet. In the initial experiments on this problem, Wistar rats fed on a commercial chow diet were used; later this work was continued with a crossed strain of Wistar piebald rats fed on a diet containing 70% wheat flour, 17% milk powder, 6% fat, 2·5% yeast, 2·5% meat, 0·9% cod-liver oil, 0·093% wheat-germ oil and 0·16% salt mixture. No striking differences in liver metabolism or ali-esterase activity were noted between the two groups. Male rats (200–250 g.) of the latter strain were used in most of the experiments reported below.

Measurement of esterase activity. As in previous investigations (cf. Mendel & Rudney, 1943; Hawkins & Mendel, 1949; Myers, 1950), the activity of the ali-esterases was measured manometrically at pH 7·4 by the Warburg method at 37·5° in a medium containing 0·025 M-$NaHCO_3$ saturated with 5% CO_2 and 95% N_2 (v/v). Liver homogenates were made with a glass Potter-Elvehjem homogenizer; 0·05 ml. of a 50-fold diluted homogenate was usually employed for determination of the ali-esterase activity of normal liver. The enzyme preparation was placed in the main compartment of the Warburg vessel, the substrate in the side arm, and the inhibitor was added to the enzyme-bicarbonate mixture in the main compartment. The total fluid volume was usually 5 ml. After equilibration of the vessels at 37·5° for 30–40 min., the substrate was tipped in from the side arm. Readings were started 2 min. later and repeated at appropriate intervals, usually every 3 or 5 min.

Esterase activity was calculated from the initial rate of hydrolysis of substrate; the rate of hydrolysis was usually constant for at least 30 min. when tributyrin or triacetin were used as substrate for the ali-esterase, but tended to diminish markedly after 10 min. when Tween 20 or Tween 80 (Honeywill and Stein, Ltd., 21 St James's Square, S.W. 1) were used. In all cases the final concentration of ester was 1% (v/v); routine determinations of ali-esterase activity were done with a tributyrin emulsion stabilized by the addition of 0·5% gum acacia (final concentration). Other esters used as substrates for the ali-esterases in this investigation were triacetin, methyl butyrate, ethyl acetoacetate, Tween 20 and Tween 80. The two last-mentioned are water-soluble lauric and oleic acid esters, respectively, of a polyoxyalkylene derivative of sorbitan (Archibald, 1946).

The esterase activity of liver slices was measured in a bicarbonate-Ringer medium; the dry weight of these slices was 3–10 mg. (depending on the esterase inhibition) and esterase values were corrected for the small initial rate of CO_2 production due to the breakdown of carbohydrate in the slices. Neither the ali-esterase activity of liver homogenate nor the degree of inhibition was affected when a simple bicarbonate medium was replaced by a bicarbonate-Ringer medium. The esterase activity was measured over a period of 10–30 min., depending on how long the initial rate of hydrolysis remained constant; but in all cases the esterase activity, after correction for non-enzymic hydrolysis of the substrate, was calculated as a Q_{ester} value, i.e. μl. CO_2 produced/mg. dry wt. of enzyme preparation/hr.

Experiments with inhibitors. Ali-esterase inhibitors used were sodium arsanilate (atoxyl), tri-*o*-cresyl phosphate (TOCP), di*iso*propyl fluorophosphonate (DFP), tetraethyl pyrophosphate (TEPP), and diethyl *p*-nitrophenyl phosphate (E600). The last three of these inhibitors had to be prepared freshly in aqueous solution for each experiment; anhydrous propylene glycol, the usual solvent for preparing stable stock solutions, could not be used since it was found that propylene glycol in concentrations of 0·2–0·5% is itself a strong inhibitor of acetoacetate production.

The TOCP used in this investigation had been purified by distillation at low pressure of a technical product (Eastman-Kodak); the resulting preparation, a clear oil ($n_D^{5.5°} = 1.5538$) had no trace of the slightly yellowish tinge usually found in less pure preparations. Pure TOCP has very little toxicity for rats (Mendel & Rudney, 1944); the ali-esterases of liver are about 70% inhibited, and that of serum almost completely, by the intramuscular injection of 5 μl. TOCP/100 g. body wt. (Mendel & Mortimer, 1944; Myers & Mendel, 1949). In the present investigation, rats of 200–250 g. were usually injected intramuscularly with 0·1 ml. TOCP every second day beginning 5 days before the rats were sacrificed. This treatment resulted in a 95–97% inhibition of the tributyrin-esterase activity of the liver.

For experiments with liver slices, the rat was killed by stunning and decapitation, the liver removed rapidly and dropped into ice-cold Ringer solution. Three slices of 25–30 mg. total dry wt. were placed in a Dickens-type respiration vessel containing 2·5 ml. cold phosphate-Ringer solution (pH 7·4). The vessel with manometer was filled with O_2, equilibrated at 37·5° for 15 min. and the rate of O_2 uptake measured over a subsequent period of 120 min. The total acetoacetate produced in the 135 min. was determined in a 2 ml. sample of the fluid medium by the method of Jowett & Quastel (1935*a*). O_2 uptake was calculated as μl. O_2 taken up/mg. dry wt. of liver slice/hr. ($-Q_{O_2}$) and acetoacetate production as μl. CO_2 formed on decarboxylation of the acetoacetate produced/mg. dry wt. of liver slice/hr. ($Q_{acetoacetate}$).

RESULTS

Ali-esterases and acetoacetate production of liver slices

Typical values obtained in individual experiments which show the effects of various ali-esterase inhibitors upon the oxygen uptake, acetoacetate production and tributyrin-esterase activity of rat-liver slices are presented in Table 1. It is found, in agreement with the results of Jowett & Quastel (1935*b*), that atoxyl causes a relatively selective inhibition of the spontaneous acetoacetate production and of the tributyrin-esterase activity. On the other hand, E600 has no effect upon acetoacetate production when present in concentrations which inhibit the tributyrin hydrolysis almost completely.

The values for percentage inhibition of ali-esterase activity and of acetoacetate production by atoxyl are very similar to those reported by Jowett & Quastel (1935*b*). The interpretation of these

Table 1. *Effect of ali-esterase inhibitors on the O_2 uptake, acetoacetate production and tributyrin-esterase activity of rat-liver slices*

(Rat-liver slices incubated in phosphate-Ringer medium at 37·5° for 135 min. with and without inhibitor in the medium. Average O_2 uptake during the period 15–135 min. is given as $-Q_{O_2}$; average acetoacetate production during the total period 0–135 min. as Q_{Ac}. Extra acetoacetate production in the presence of sodium butyrate is calculated from the differences in Q_{Ac} values in the presence and absence of butyrate. The tributyrin-esterase activity of slices from the same liver was measured in bicarbonate-Ringer medium as described in the text, with and without inhibitor in the medium; average calculated as Q_{TB}. Typical results in experiments with five different esterase inhibitors are given below. The values for percentage inhibition by $1·2 \times 10^{-2}$ M-atoxyl, as calculated from the results obtained by Jowett & Quastel (1935*b*) with a similar method, are given in brackets.)

	In absence of butyrate				In presence of 0·01 M-sodium butyrate							
									Extra			
Inhibitor and concentration	$-Q_{O_2}$ (μl./mg. dry wt./hr.)	Inhibition (%)	Q_{Ac} (μl./mg. dry wt./hr.)	Inhibition (%)	Q_{O_2} (μl./mg. dry wt./hr.)	Inhibition (%)	Q_{Ac} (μl./mg. dry wt./hr.)	Inhibition (%)	Q_{Ac} (μl./mg. dry wt./hr.)	Inhibition (%)	Q_{TB} (μl./mg. dry wt./hr.)	Inhibition (%)
None	10·2	—	0·68	—	14·7	—	1·74	—	1·06	—	141·8	—
10^{-2} M-Atoxyl	8·7	15 (37)	0·13	81 (80)	9·8	33 (35)	1·11	36 (37)	0·98	8 (28)	41·3	71 (74)
None	11·4	—	0·80	—	15·9	—	2·04	—	1·24	—	148·5	—
10^{-4} M-E 600	12·0	−5	0·81	−1	16·1	−1	2·07	−2	1·26	−2	2·4	98
None	12·3	—	1·01	—	15·2	—	3·50	—	2·49	—	162·4	—
10^{-6} M-DFP	11·8	4	0·98	3	15·9	−5	3·50	0	2·52	−1	29·2	82
None	11·4	—	0·72	—	—	—	—	—	—	—	168·5	—
10^{-4} M-TEPP	11·7	−3	0·71	1	—	—	—	—	—	—	10·3	94
None	12·9	—	0·75	—	—	—	—	—	—	—	111·5	—
Approx. 5×10^{-4} M-TOCP (sat.)	12·9	0	0·76	−1	—	—	—	—	—	—	45·1	60

results is complicated by the fact that atoxyl causes a progressive inhibition of the respiration of the liver slices, the Q_{O_2} values given in Table 1 being an average of the results obtained during the 2 hr. period of incubation. The effect that this inhibition of respiration might have upon acetoacetate production is not known; this point will be discussed later.

The tributyrin-esterase activity is strongly inhibited by low concentrations of TEPP, DFP, TOCP and E 600; with none of these inhibitors was there any appreciable effect on the rate of oxygen uptake or the spontaneous acetoacetate production of the liver slices. The other compounds were less suitable than E 600. TEPP is much less stable than E 600 under the experimental conditions. The maximal inhibition of ali-esterase activity by TOCP *in vitro* was usually less than 60 %, whereas a 98–99 % inhibition was readily obtainable with E 600. Both respiration and acetoacetate production were depressed when the ali-esterase was inhibited more than 75 % by DFP.

Thus E 600 seemed to be the best choice as an innocuous *in vitro* inhibitor of the ali-esterase activity of rat-liver slices and most of the subsequent experiments with liver slices were therefore carried out with this compound. Although the results of individual experiments showed small differences in the acetoacetate production and oxygen uptake, whether E 600 was present or not, no definite trend could be observed which would indicate that these variations were due to esterase inhibition (Table 5). The average results of eighteen experiments showed no significant difference between the rate of oxygen uptake, or of acetoacetate production, in the presence ($Q_{O_2} = -11·16$; $Q_{acetoacetate} = 0·78$) and in the absence ($Q_{O_2} = -11·14$; $Q_{acetoacetate} = 0·75$) of an initial concentration (10^{-4} M) of E 600 which inhibited the ali-esterase activity of the slices by 98 %. The small variations of less than 5 % in the results of individual experiments with and without E 600 are attributable to experimental errors.

These results would seem to indicate, first, that the ali-esterases are not essential for any step in the lipid catabolism of surviving liver slices, and secondly, that the effect of atoxyl on the spontaneous acetoacetate production must be explained upon some other basis than the inhibition of ali-esterase activity. However, the possibility of methodological errors had to be taken into account before the above conclusions could be drawn. The possibilities considered are given in detail below.

(1) An enzyme catalysing the hydrolysis of DFP is present in rat liver (Mazur, 1946). Since E 600 and the other phosphate inhibitors might also be hydrolysed by the same or a similar enzyme, it was necessary to ascertain whether the ali-esterases of the liver slices remain inhibited throughout the period of incubation in the respiration vessels.

Therefore, after the 135 min. period of incubation with 10^{-4} M-E600, the slices were removed from the respiration vessels, rinsed well, and the tributyrin-esterase activity was determined in bicarbonate-Ringer solution in the absence of inhibitor. The esterase in these slices was found to be inhibited 93% when compared to the esterase activity of control slices. Since an initial concentration of 10^{-4} M-E600 caused a 98% inhibition, the average ali-esterase inhibition in the liver slices during the period of respiration measurements must have been at least 95–96%. In the following section it will be shown that even this estimate is probably too low.

(2) The tributyrin-esterase activity of a liver homogenate is usually 6–7 times greater than the esterase activity of slices from the same liver (Tables 2 and 5). Thus the possibility existed that the tributyrin hydrolysis by liver slices represented only the esterase activity of the broken exterior cells of the slice, and that the ali-esterases within the intact cells were not affected by the alkyl phosphates. To determine whether this was the case, liver slices were allowed to stand in phosphate-Ringer solution with and without 10^{-4} M-E600 for 0·5 hr. at room temperature (18°) and were then washed thoroughly during another 0·5 hr. to remove any free E600 from the slices. This procedure was used to minimize any loss of ali-esterases from the control slices (cf. Rona & Lasnitzki, 1926; Jowett & Quastel, 1935*b*). After the slices were washed five times in 50 ml. of phosphate-Ringer stirred by bubbling O_2 through the solution, some of them were used directly for measurement of esterase activity; others were first ground with water in a close-fitting Potter homogenizer and the resulting homogenate was used for measurement of esterase activity.

The ali-esterase activity of the washed slices was found to have been 88% inhibited by incubation with E600, which would seem to indicate that the irreversible combination of E600 with the ali-esterase was still incomplete after 0·5 hr. at 18°. (The inhibition of the tributyrin activity of liver homogenate by E600 approaches completion within a few min. at 37·5° and is not reversible by dilution.) A homogenate of the same washed slices showed 96% inhibition of the ali-esterase activity in place of the 88% observed with the slices (Table 2). Thus E600 inhibits the esterase in the intact cells throughout the liver slice. It would also appear that the inhibition of the ali-esterases in the liver cells must actually be significantly greater than it would appear to be from measurements on the slice itself. This latter fact can probably be explained by the low solubility of the substrate and its slow diffusion into the intact cells, factors which limit the amount of substrate available to the ali-esterase and thus limit the maximal rate of hydrolysis in normal liver slices. From these results (Table 2) it can be concluded that the inhibition of the ali-esterase in a liver slice by an initial concentration of 10^{-4} M-E600 is probably greater than 99%.

(3) The amount of acetoacetate which accumulates in the fluid medium represents the balance between the rate of production from fatty acid oxidation and the rate of further breakdown of the acetoacetate in the slices. The further oxidation of acetoacetate can be selectively inhibited to a large extent by addition of 0·02 M-malonate to the medium (Jowett & Quastel, 1935*b*). Nevertheless, in the presence of malonate the same value for spontaneous $Q_{\text{acetoacetate}}$ was obtained with or without E600 (Table 3). Thus it seems unlikely that E600 itself might possibly be inhibiting

Table 2. *Comparison of the ali-esterase activities of rat-liver slices and liver homogenate*

(Washed E600-treated and control slices were incubated in phosphate-Ringer solution with and without 10^{-4} M-E600, and subsequently washed in phosphate-Ringer solution as described in the text. The tributyrin-esterase activity of the control slices was determined in bicarbonate-Ringer solution, with and without 10^{-4} M-E600; the tributyrin-esterase activity of the washed E600-treated slices similarly, without any added inhibitor. TOCP slices were obtained from the liver of a rat which had been injected with 0·1 ml. of TOCP intramuscularly 1, 3 and 5 days before the rat was killed. A portion of the slices was ground with water in each case and the resulting homogenate was also tested for tributyrin-esterase activity, all esterase values being expressed as Q_{TB}.)

	Slices		Homogenate of similar slices	
Material	Q_{TB} (μl./mg. dry wt./hr.)	Inhibition of esterase activity (%)	Q_{TB} (μl./mg. dry wt./hr.)	Inhibition of esterase activity (%)
Washed control slices	193	—	1282	—
Washed control slices + 10^{-4} M-E600	(4)	(98)	—	—
Washed E600-treated slices	23	88	55	96
TOCP slices	31	84	62	95

Table 3. *Effect of sodium malonate on the acetoacetate production of rat-liver slices in the presence and absence of ali-esterase inhibitor*

(Oxygen uptake (Q_{O_2}) and acetoacetate production (Q_{Ac}) of liver slices measured in phosphate-Ringer medium as in Table 1. TOCP slices obtained from the liver of a rat injected intramuscularly with TOCP as in Table 2.)

	Control values		Average values in the presence of 0·02 M-sodium malonate	
Material	$-Q_{O_2}$	Q_{Ac} (μl./mg. dry wt./hr.)	$-Q_{O_2}$	Q_{Ac}
Normal slices	10·4	0·92	8·8	2·16
Normal slices + 10^{-4} M-E600	10·3	0·89	9·1	2·21
TOCP slices	12·0	0·83	9·2	2·05

simultaneously both acetoacetate formation and acetoacetate oxidation to the same extent.

(4) Previous evidence has indicated that the activity of beef- and pig-liver homogenates towards tributyrin might represent the activity of two different forms of ali-esterase (Fodor, 1946; Falconer & Taylor, 1946). In the present investigation it was found possible to obtain a selective inhibition of the esterase activities of rat-liver homogenate towards different aliphatic esters (Table 4). These results can only be explained by the assumption that rat-liver homogenate contains three different ali-esterases; their relative substrate specificities appear to overlap, but all three seem to have the characteristics of an ali-esterase.

The presence of more than one ali-esterase in the liver made it necessary to determine whether E600 inhibits the esterase activities towards all of these different aliphatic esters. This was found to be true (Table 5), although slight differences in sensitivity to inhibition by E600 were observed.

(5) Evans (1951) has shown that the ali-esterases of bone marrow hydrolyse acetoacetate esters. This would suggest that such an esterase might play an important role in the metabolism of acetoacetate. Ethyl acetoacetate was also hydrolysed by liver ali-esterase, but the esterase activity towards this acetoacetate ester was inhibited by E600 just as well as the activity towards tributyrin (Table 5).

The conclusion that the ali-esterases are not required for any step in the acetoacetate production of liver cells was corroborated by experiments in which the liver esterases were inhibited *in vivo* by the intramuscular injection of TOCP into rats. The degree of esterase inhibition in the liver is again greater when the inhibition is measured in homogenates as compared with that measured in slices (Table 2); however, the inhibition of ali-esterase is never as great as that obtainable with E600 *in vitro*. The tributyrin-esterase activity of liver homogenate is reduced to about 5% of the normal level (Tables 2 and 4) and there is no appreciable change in the rate of oxygen uptake or acetoacetate production of liver slices (Table 3) despite the fact that the ali-esterases had been inhibited *in vivo* for 5 days.

Although these experiments show that ali-esterase activity is not essential for the catabolism of the lipids in isolated liver slices, nevertheless

Table 4. *Experiments on the inhibition of the various esterase activities of rat-liver homogenate*

(Rat-liver homogenate containing 1 part by weight of fresh liver plus 9 vol. of water was exposed to high temperature or alkaline pH; the esterase activities towards different substrates were measured in bicarbonate buffer and percentage inactivation calculated by comparison with the esterase activities of the original homogenate. The inhibitory effect of TOCP *in vitro* was measured by incubating the liver homogenate with 0·005% TOCP in the bicarbonate buffer for 30 min. before determining the esterase activities. The inhibition of esterase activities caused by injection of TOCP *in vivo* was determined by comparison of normal Q values with those obtained on liver homogenates from three rats injected intramuscularly with TOCP as in Table 2.)

	Amount of the esterase activity remaining			
Substrate	After exposure of the liver homogenate to 62° for 10 min. (%)	After exposure of the liver homogenate to pH 10 for 40 min. (%)	After incubation with 0·005% TOCP *in vitro* (%)	After injection of TOCP into the rat (%)
Triacetin	6	59	68	14
Tributyrin	9	76	46	5
Tween 20	54	91	52	4
Tween 80	74	100	80	4

Table 5. *Effect of various concentrations of E600 upon the ali-esterase activities and acetoacetate production of rat liver*

(Esterase activities of twelve different liver homogenates were measured in a bicarbonate buffer and expressed as average Q_{ester} values for various esters present in a concentration of 1% (v/v). The effect of E600 upon the esterase activity was determined after incubation with E600 in the bicarbonate buffer for 30 min. before the substrate was added. Oxygen uptake, acetoacetate production and tributyrin-esterase activity of slices from one of the livers were determined as in Table 1.)

	Average Q_{ester} values of liver homogenate with the following esters					Liver slices in phosphate-Ringer		
Concentration of E600 (M)	Triacetin	Tributyrin	Ethyl acetoacetate	Tween 20	Tween 80	Q_{TB}	$-Q_{O_2}$	Q_{Ac}
	(μl./mg. dry wt./hr.)							
0	551	828	164	68	23	142	10·2	0·60
10^{-8}	485	670	123	56	20	137	10·2	0·62
10^{-7}	—	49	8·9	—	—	105	10·1	0·61
10^{-6}	50	33	6·2	2·0	1·6	55	10·1	0·65
10^{-5}	—	8·1	1·9	—	—	11	10·1	0·59
10^{-4}	7·0	0·8	0·0	0·4	0·2	3	10·3	0·64

synthetic fatty acid esters added to the medium were not catabolized unless they were first hydrolysed by the esterases.

Small amounts (0·01%, i.e. 0·001 M) of the water-soluble ester methyl butyrate were added to the phosphate-Ringer solution in the respiration vessels and incubated with liver slices for 60 min. with and without E600. (Higher concentrations of methyl butyrate could not be used because the hydrolysis of this ester was rapid enough to produce excessive free acid in the cells.)

Under these conditions, the presence of methyl butyrate causes an increase in acetoacetate production (Table 6); methyl butyrate was even more effective than was sodium butyrate. However, methyl butyrate has little effect on acetoacetate production when the hydrolysis of this ester by ali-esterase is inhibited by addition of E600 to the medium. Thus it seems justified to conclude that hydrolysis of methyl butyrate is essential before acetoacetate can be formed from the butyrate residue. Experiments with the water-soluble lauric acid ester Tween 20 gave similar results (Table 6).

Table 6. *Effect of E600 on the oxidation of fatty acid esters by rat-liver slices*

(Rat-liver slices were allowed to stand 30 min. at 20° in Ringer solution with and without 10^{-4} M-E600. The same slices were then incubated in phosphate-Ringer medium at 37·5° for 60 min. with and without inhibitor in the medium. $-Q_{O_2}$ was calculated from the average O_2 uptake during the period 15–60 min.; Q_{Ac} from the average acetoacetate production during the total period 0–60 min. Sodium butyrate, methyl butyrate or Tween 20 were added to some of the respiration vessels as substrate for the fatty acid oxidative enzymes.)

Added substrate	Control values		Values obtained with 10^{-4} M-E600 in the medium	
	$-Q_{O_2}$	Q_{Ac}	$-Q_{O_2}$	Q_{Ac}
	(μl./mg. dry wt./hr.)			
None	11·5	0·62	10·9	0·65
0·001 M-Sodium butyrate	13·8	1·56	13·7	1·60
0·001 M-Methyl butyrate	14·5	1·98	11·6	0·71
None	10·6	0·84	10·1	0·80
0·4% Tween 20	11·9	1·67	10·8	1·08

From this last experiment it might also be presumed that the constituent fatty acids of the liver lipids can be oxidized only after the lipids have been hydrolysed. Inhibition of ali-esterase activity, whether by E600 or by atoxyl, does not affect lipid catabolism in the liver cells; nevertheless, atoxyl does inhibit the catabolism of liver lipids although it does not interfere appreciably with the oxidation of free fatty acids. Thus it seemed logical to suppose that atoxyl inhibits the hydrolysis of the liver lipids, and that the enzyme responsible for the hydrolysis of liver lipids is distinct from the ali-esterases.

Lipid hydrolysis in liver homogenates

Fairbairn (1945*b*) has shown that a fairly rapid liberation of free fatty acids occurs in liver homogenates and indicated that this was probably due to hydrolysis of phospholipins. Other investigators have found that the total amount of phospholipins decreases during incubation of liver slices (Fishler, Taurog, Perlman & Chaikoff, 1941; Sperry, Brand & Copenhaven, 1942). Thus it was of interest to determine what effect the ali-esterase inhibitors would have upon phospholipin hydrolysis and whether the differences in the effects of E600 and atoxyl upon spontaneous acetoacetate production could be explained upon this basis.

To determine the rate of lipid hydrolysis it was necessary to use a liver homogenate since phospholipin is not only hydrolysed but also re-synthesized in liver slices (Fishler *et al.* 1941), and since the free fatty acids liberated by lipid hydrolysis would be catabolized by intact liver cells. Several experiments were performed using a procedure similar to the example described below. The methods of lipid analysis were based upon those recommended by Bloor (1943) and Fairbairn (1945*a*).

Three adult male rats of the Wistar-piebald strain were killed by stunning and decapitation; the livers were dissected out immediately and dropped into a large volume of ice-cold Ringer solution. The livers (approx. 25 g.) were then weighed and homogenized with 100 ml. of cold phosphate buffer (0·143 M, pH 7·4) for 30 sec. in a Waring Blendor. Two 10 ml. samples of the suspension were each pipetted into 70 ml. of boiling ethanol; other portions of the suspension were added to a small volume of water or inhibitor solution in a conical flask, mixed thoroughly and placed in a water bath at 37·5°. The whole procedure from the time of killing the first rat to this point took less than 5 min. Samples were then removed from the incubated suspensions for determination of pH, ali-esterase activity and lipid content.

The ethanolic suspensions were kept just below boiling for 30 min., filtered, the residue re-extracted with smaller volumes of ethanol, the combined filtrates evaporated to remove the ethanol and re-extracted with light petroleum (Bloor, 1943). Acetone-soluble and acetone-insoluble fractions were isolated and the free fatty acids of the acetone-soluble fraction were determined by the method of Fairbairn (1945*a*). The total fatty acid in the same sample

was subsequently determined after hydrolysis of the esterified lipids with alkali. All lipid values were expressed as m-moles of fatty acid/g. dry wt. of liver.

The results obtained in five such experiments are shown in Table 7. The lipid composition in the livers from rats which had been injected with TOCP for 5 days before they were killed was well within the range of normal values. In other words, 97% inhibition of the ali-esterase activity of rat liver *in vivo* had no marked effect upon the liver lipids in a short-term experiment.

The rate of liberation of free fatty acids by hydrolysis of the lipids in the liver homogenates was fairly well reproducible from one experiment to another (Table 8), and was not affected significantly when the ali-esterase activity was inhibited 80–90% by addition of DFP or E600 to the liver homogenate. (The esterase inhibition by DFP and E600 is somewhat less than previously noted in Tables 1, 2 and 5, due probably to the use of much more concentrated liver suspensions in this case. Undoubtedly more inhibitor would be needed to inhibit the esterase in more concentrated suspensions, but a second factor which appears is the progressive decrease in inhibition during incubation of the concentrated liver homogenates. This phenomenon was not found to occur to an appreciable extent during incubation of liver slices or very dilute liver suspensions, suggesting that concentrated liver suspensions contain a factor which catalyses the destruction of combined inhibitor.) The ali-esterase activity of a homogenate of livers from rats injected with TOCP remained almost completely inhibited throughout the 3 hr. period of incubation and again the rate of lipid hydrolysis was within the normal range.

Atoxyl is the only one of the ali-esterase inhibitors tested which inhibits ali-esterase activity and lipid hydrolysis to a similar extent, just as atoxyl was the only inhibitor which inhibited ali-esterase activity

Table 7. *Lipid composition of rat-liver homogenate*

(Lipid analyses as described in the text. Each analysis was performed on a combined homogenate of three rat livers. Exps. 1–3 were performed with livers from normal rats; exps. 4 and 5 with the livers obtained from rats which had been injected intramuscularly with 0·4 ml. of TOCP spread over a period of 5 days, beginning with an initial dose of 0·2 ml. followed by two injections of 0·1 ml. on the third and fifth days.)

	Lipid content of normal rat liver				Lipid content of livers from rats injected with TOCP		
		Acetone-soluble lipids			Acetone-soluble lipids		
Exp. no.	Acetone-insoluble lipids	Esterified fatty acid	Free fatty acid	Exp. no.	Acetone-insoluble lipids	Esterified fatty acid	Free fatty acid
	(m-moles fatty acid/g. dry wt. of liver)				(m-moles fatty acid/g. dry wt. of liver)		
1	0·156	0·069	0·008	4	0·189	0·089	0·015
2	0·171	0·076	0·012	5	0·165	0·078	0·009
3	0·198	0·113	0·007				

Table 8. *Liberation of free fatty acids during incubation of the liver homogenate in the presence and absence of ali-esterase inhibitors*

(The above liver homogenates were incubated at 37·5° in the presence and absence of ali-esterase inhibitors; pH of the homogenate was 7·4 at 0 min. and 7·2 after 180 min. incubation. The free fatty acids contained in a 10 ml. sample of homogenate were determined after 180 min. by the method of Fairbairn (1945*a*). Ali-esterase activity of the homogenate was measured in a bicarbonate buffer without further addition of inhibitor.)

		Ali-esterase activity of the liver homogenate (Q_{TB})				Change in composition of the lipids of the liver homogenate after 180 min. incubation (as m-moles fatty acid/g. dry wt. of liver)			
Exp. no.	Added inhibitor	15 min. incubation	70 min. incubation	180 min. incubation	Average inhibition (%)	Increase in free fatty acid	Inhibition (%)	Decrease in neutral lipid	Decrease in phospholipin
1	None	766	736	716	—	0·053	—	0·011	0·042
	10^{-2} M-Atoxyl	272	272	278	63	0·029	45	0·012	0·016
	10^{-4} M-DFP	70	117	191	83	0·053	0	0·011	0·041
2	None	821	783	748	—	0·045	—	0·009	0·036
	10^{-4} M-E600	30	61	177	89	0·041	9	0·007	0·035
3	None	707	674	647	—	0·042	—	—	—
	Approx. 5×10^{-4} M-TOCP (sat.)	312	345	373	49	0·043	0	—	—
4	TOCP liver	25	25	22	(97)	0·042	—	—	—

and spontaneous acetoacetate production of liver slices to approximately the same extent. A study of the lipid changes involved (Table 8) shows that most of the free fatty acid liberated arises from the hydrolysis of phospholipin, as suggested by Fairbairn (1945*b*), although the hydrolysis of neutral fat contributes about 20% to the total free fatty acids under these conditions. The total fatty acid of the three fractions (phospholipin plus neutral lipid plus free fatty acid) remained fairly constant in all of the determinations; this confirms the reliability of the methods used and excludes the possibility that free fatty acids might be destroyed in the buffered liver homogenates. It appears from Table 8 that there must be at least two different enzymes involved in the hydrolysis of liver lipids to free fatty acid, a lipase and a phospholipase; both of these lipid-hydrolysing enzymes are distinct from any E600- or DFP-sensitive ali-esterase in the liver suspension. The lipase activity is not significantly affected by atoxyl in the concentrations used, while the phospholipin hydrolysis is inhibited to about the same extent by atoxyl as is the total ali-esterase activity. This latter result may explain the inhibition of the spontaneous acetoacetate production of liver slices by atoxyl.

DISCUSSION

The pertinent data concerning the selectivity of inhibition of various biochemical reactions by atoxyl and the alkyl phosphate derivatives (E600 and DFP) are summarized in Table 9.

DFP and E600 are selective inhibitors of the ali-esterases in rat liver and have little or no effect upon oxygen uptake, spontaneous acetoacetate production, oxidation of butyrate to acetoacetate, phospholipin hydrolysis, or neutral-lipid hydrolysis. Atoxyl also inhibits ali-esterases, but this inhibition could not be responsible for the inhibition of spontaneous acetoacetate production by atoxyl, since no interference with acetoacetate production is seen when the ali-esterases are almost completely inhibited by E600 or DFP. However, in contrast to DFP and E600, atoxyl inhibits not only the ali-esterase but also the phospholipase activity. From these results and from the other data obtained in this investigation, it seems justifiable to conclude that the inhibition of the spontaneous acetoacetate production by atoxyl is due to the inhibition of phospholipin hydrolysis.

As is true for most enzyme inhibitors, neither of these types of inhibitor is absolutely specific for any one enzyme or for any one metabolic process; the above concentrations proved to be about the best for demonstrating the described selective effects. E600 has very little effect on the oxygen uptake of liver slices even when present in high concentrations, but 2×10^{-4}M-DFP does cause an appreciable inhibition of the respiration of liver slices (cf. Brooks, Ransmeier & Gerard, 1949). The range of concentrations of atoxyl in which a definitely selective inhibition of spontaneous acetoacetate production can be demonstrated is fairly small (cf. Jowett & Quastel, 1935*b*). Atoxyl (10^{-2}M) does inhibit the oxygen uptake of liver slices a little but inhibits the spontaneous acetoacetate production to a much greater extent; slightly higher concentrations (e.g. 2×10^{-2}M) cause a very appreciable inhibition of the rate of oxygen uptake and the selective effect upon spontaneous acetoacetate production is no longer clear-cut. It is not certain whether this inhibition of respiration is due in part to an inhibition of lipid oxidation or whether it merely reflects the ability of atoxyl and DFP in high concentrations to combine non-specifically with a great many types of enzymic and non-enzymic proteins. If we assume the latter to be true, then it would be necessary to make a small correction in the acetoacetate production for the inhibition of general respiration.

Another factor which has to be taken into consideration is the possibility that less of the natural substrates in the liver cells would be oxidized in the

Table 9. *Summary of data on selectivity of inhibition of various biochemical reactions by atoxyl and the alkyl phosphate derivatives (E600 and DFP)*

	Inhibition by atoxyl (10^{-2}M) (%)	Inhibition by E600 (10^{-4}M) (%)	Inhibition by DFP (10^{-6}M) (%)
Liver slices (data from Table 1):			
Oxygen uptake	15	0	0
Spontaneous acetoacetate production	81	0	0
Extra acetoacetate production in the presence of 0·01 M-sodium butyrate	8	0	0
Ali-esterase activity	71	98	82
Liver homogenate (data from Table 7):	(10^{-2}M)	(10^{-4}M)	(10^{-4}M)
Ali-esterase activity	63	89	83
Phospholipin hydrolysis	62	0	0
Neutral lipid hydrolysis	0	0	0

presence of the salt of a more readily oxidizable free fatty acid such as sodium butyrate. These problems could probably be answered most effectively by the use of isolated enzyme systems capable of oxidizing various forms of esterified and free fatty acids. The necessary corrections of the data given above would be relatively small in the case of $Q_{acetoacetate}$ values, where the addition of sodium butyrate causes an increase of 150–250%; the overall rate of oxygen uptake of liver slices, however, is increased only by 25–45% on addition of sodium butyrate and thus these values are less reliable criteria.

The experimental data obtained in this investigation seem sufficiently clear-cut to permit several significant conclusions. They fail to give any indication of an essential function of ali-esterases in the normal oxidative processes of rat-liver cells. On the other hand, they have tended to corroborate the generally accepted theories regarding lipid oxidation in liver (Jowett & Quastel, 1935*b*; Stadie, 1945).

It appears that the lipids of rat liver must be hydrolysed before the constituent fatty acids can be oxidized, or, at least, before their oxidation can give rise to free acetoacetate. This is indicated by the results with synthetic esters as presented in Table 6, and by the fact that atoxyl inhibits the spontaneous acetoacetate production of liver slices when present in a concentration which also inhibits the hydrolysis of liver lipids. It also seems probable that the phospholipins must play an important role in the lipid catabolism of normal liver. Most of the free fatty acid liberated by lipid hydrolysis in liver homogenates is derived from the phospholipins, and a concentration of atoxyl which selectively inhibits phospholipin hydrolysis (as opposed to the hydrolysis of neutral fat) also inhibits selectively the spontaneous acetoacetate production of liver cells (as opposed to the extra acetoacetate production in the presence of a free fatty acid).

Thus the main pathway of lipid oxidation in normal rat liver would involve, first, hydrolysis of phospholipin and, secondly, oxidation of the free fatty acids liberated. If this conclusion is justified, it would automatically eliminate ω-oxidation (Verkade & van der Lee, 1934) or oxidation of unsaturated bonds (Hove, 1943) as a significant source of free acetoacetate from esterified fatty acid. Thus it would not be expected that ephemeral lipid esters containing short-chain fatty acids would ever be produced in the normal lipid catabolism of liver, and consequently ali-esterase activity should not be of importance in lipid catabolism.

This conclusion seems justified by the fact that the liver ali-esterases can be almost completely inhibited *in vitro* or *in vivo* without any effect upon either the acetoacetate production or the total oxygen uptake of liver slices. The fact that the ali-esterases also hydrolyse acetoacetate esters would seem to be merely incidental; these esterases have no absolute substrate specificity and will hydrolyse a wide variety of synthetic esters structurally related to those upon which they act preferentially.

Ali-esterase inhibition *in vivo* does not cause any marked change in the composition of the liver lipids in short-term experiments, and does not affect the regeneration of liver adversely (Mendel, Myers & Simons, 1952). Thus our investigations have not disclosed the primary functional role or physiological substrate of the large amount of ali-esterase in liver. The possible identity of certain types of ali-esterase with other hydrolytic enzymes such as cholesterol esterase (cf. Swell, Cassidy & Treadwell, 1951) or some amidases remains to be investigated.

SUMMARY

1. The ali-esterases of rat liver are highly sensitive to inhibition by various alkyl phosphate derivatives such as diethyl *p*-nitrophenyl phosphate (E600). They can be selectively inhibited *in vivo* by intramuscular injection of tri-*o*-cresyl phosphate.

2. Both the ali-esterases and the phospholipase are inhibited 60–70% by 10^{-2}M-atoxyl. The lipase activity is not significantly affected either by the phosphate derivatives or by atoxyl in concentrations which inhibit ali-esterase activity.

3. The spontaneous acetoacetate production, ali-esterase activity and phospholipid hydrolysis of liver are all inhibited to a similar extent by atoxyl. Selective inhibition of ali-esterases by the phosphate derivatives *in vitro* or *in vivo* has no effect upon the acetoacetate production or oxygen uptake of liver slices.

4. The results fail to give any indication of an essential function of ali-esterases in the normal lipid metabolism of rat liver.

5. The results tend to confirm the generally accepted theories regarding lipid oxidation in liver. The main pathway of lipid oxidation would involve, first, hydrolysis of phospholipin, and secondly, oxidation of the free fatty acids liberated.

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Amino-acid Utilization in Bacterial Growth

1. PEPTIDE UTILIZATION BY A LEUCINE-REQUIRING MUTANT OF *ESCHERICHIA COLI*

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In the course of studies on the quantitative determination of amino-acids with lactic acid bacteria, numerous authors have studied the availability of peptides in place of the corresponding amino-acids. In all cases so far published, it has been found that the peptides either show no activity, or an activity inferior or equal to that of the constituent amino-acids. The magnitude of the response of the organisms depended on the nature of the amino-acids (other than the one missing in the assay) present in the peptide and on their sequence in the peptide molecule.

In two cases only, a growth response greater with peptides than with the sum of equimolar quantities of the constituent amino-acids has been found. This effect has been observed (*a*) with the strain SF, a member of the genus *Pseudomonas*, studied by Simmonds & Fruton (1949, 1950, 1951); (*b*) with the two proline-requiring mutants of *Escherichia coli* (K 12–679–183 and 58–6317), studied by the same authors (Simmonds & Fruton, 1948). In the case of the strain 'SF', the organism can grow on leucine or glycine as the sole source of carbon and nitrogen, but a better growth is obtained on equimolar amounts of L-leucylglycine. However, the organism can also be grown on a variety of amino-acids as nitrogen source, such as L-phenylalanine, L-alanine, L-glutamate, L-isoleucine and L-valine. Thus, this organism exhibits no specificity as to its amino-acid requirement.

On the contrary, the proline-requiring mutants of *Esch. coli* present an absolute requirement for proline. A number of proline dipeptides and tripeptides show growth-promoting activity 1·5–3

times that of proline or of proline plus the corresponding amino-acid (or acids), independently of the nature of the other amino-acids and of their sequence in the molecule.

These two different cases seem to invalidate the view previously expressed by Simmonds, Tatum & Fruton (1947), that peptides must be hydrolysed to the corresponding amino-acids prior to their utilization for growth. This view was based in part on the fact that the leucine-requiring mutant K 12–679–680 does not give a better growth response with leucine peptides than with leucine alone or leucine plus the corresponding amino-acids.

In the present paper, we describe a genotypically leucine-requiring mutant which can be rendered phenotypically leucine-peptide requiring. This was accomplished with a number of natural inhibitors which seem to act in competitively inhibiting the incorporation of leucine into peptides. The results of our experiments suggest that, at least in the presence of these inhibitors, the peptides of leucine are not utilized through mere hydrolysis.

We should like to emphasize the importance of antagonisms between amino-acids in the growth of micro-organisms. These antagonisms may be regarded as regulating the rate of synthesis of various amino-acids and peptides. Such regulations may possibly help to ensure the synthesis of specific protein structures.

EXPERIMENTAL

Strains utilized. Strain ML 328*c*, a leucine-requiring strain of *Esch. coli*, obtained by the penicillin selection method of Davis (1949, 1950), has been utilized. This strain was obtained by Monod (unpublished) from strain ML3. We wish to thank Dr Monod for supplying us with this mutant and the parent strain. The organism was maintained on meat-broth agar slants by weekly transfers.

Testing methods. The assays were conducted in 16 × 1·8 cm. tubes. The liquid volume was 5·0 ml. The medium used was that of Monod (1942), containing 5 g. glucose/l.; 3 ml. of this medium were added to each tube and the volume made up to 5 ml. by the addition of the inoculum, other substances and water. All the substances except the α-keto- and α-hydroxy-γ-methyl-*n*-valeric acid and the peptides were added prior to sterilization, which was effected in the autoclave at 115° for 20 min. The α-keto-γ-methyl-*n*-valeric acid, α-hydroxy-γ-methyl-*n*-valeric acid and the peptides were sterilized by filtration and added after autoclaving. The tubes were then cooled and inoculated. The inoculum was prepared in the following manner: a loopful of organisms from an 18 hr. slant was suspended in 10 ml. of sterile water and brought to a standard turbidity. The suspension was then diluted 100-fold and 0·5 ml. of this final suspension was added to each tube. This corresponds roughly to an inoculum of 10^6 cells per tube. The contents of the tubes were then mixed and the tubes incubated at 37° in an upright position without shaking for periods of 17–24 hr., according to the experiment. At the end of this period, growth was estimated turbidimetrically with the Meunier electrophotometer (green filter); 100 divisions of the drum correspond to $1·3 \times 10^8$ cells per ml. Under these conditions, maximal growth of the parent strain ML3 corresponds to 150 divisions (18 hr. period). The mutant was stable and no prototrophic growth was noted in the tubes without added leucine, even after several days.

Materials. L-Leucine, L- and D-isoleucine, L- and D-valine, DL-serine, DL-alanine, L-arginine, L-ornithine, DL-lysine, L-tyrosine, L-phenylalanine, L-histidine, L-proline, L-glutamic and aspartic acids, DL-threonine, glycine, synthetic DL-norleucine, and all the peptides were Hoffmann-La Roche products. Synthetic DL-methionine and DL-tryptophan were gifts from L'Alimentation Equilibrée.

α-Keto-γ-methyl-*n*-valeric acid was prepared by using ophio-L-amino-acid-oxidase of the venom of *Vipera aspis* acting on L-leucine, according to Singer & Kearney (1950). We thank Dr P. Boquet who supplied us generously with the snake venom. α-Hydroxy-γ-methyl-*n*-valeric acid was a generous gift from Dr D. M. Bonner, to whom our thanks are due.

RESULTS

Leucine requirement of the leucine-requiring mutant

Under the conditions defined above, the concentration of L-leucine required to produce half-maximal growth of strain ML328*c* is 7×10^{-6}M (incubation time, 18 hr.). Under the same conditions, the concentration of α-keto-γ-methyl-*n*-valeric acid required to produce half-maximal growth is also 7×10^{-6}M.

The mutant shows poor growth even after 40 hr. with α-hydroxy-γ-methyl-*n*-valeric acid at a concentration of 10^{-4}M. The factor limiting its utilization may well be the rate of its conversion to the α-keto derivative (see Holden, Wildman & Snell, 1951). Since identical growth is obtained with L-leucine and identical concentrations of DL-leucine (expressed in L), it is assumed that D-leucine does not promote the growth of the mutant.

Action of amino-acids other than L-*leucine in the culture medium*

No inhibition of growth of the parent strain ML3 is noted in the presence of added naturally-occurring amino-acids. Cohen, Hirsch & Raynaud (1951) have already found that the growth of a valine-requiring mutant of strain ML3 is inhibited competitively by a number of amino-acids, among which isoleucine is the most potent.

The following amino-acids were tested as inhibitors of the growth of the leucine-requiring mutant ML328*c* and were found not to suppress the growth at concentrations up to 10^{-2}M: DL-alanine, L-glutamate, L-aspartate, L-arginine, L-ornithine, DL-lysine, L-tyrosine, DL-tryptophan, L-proline, L-phenylalanine and glycine. The L-leucine concentration was 10^{-5}M.

The following amino-acids, at the concentrations given in parentheses, totally suppress the growth of

the mutant (L-leucine concentration as above): DL-norleucine ($2{\cdot}5 \times 10^{-4}$M), L-isoleucine (10^{-3}M), L-valine (10^{-2}M) and DL-methionine (10^{-2}M). Irregular results were obtained with DL-serine (10^{-2}M). The concentrations given for the racemic compounds are those of the L-isomer.

Study of the inhibition of the growth of the leucine-requiring mutant by L-isoleucine

Due to the high inhibition index of methionine and valine and to the fact that the concentration of L-leucine could not be lowered below 10^{-5}M without affecting growth in the controls, a thorough study of the inhibition of growth by these two amino-acids could not be made. The case of the inhibition of the growth of the leucine-requiring mutant by norleucine, which inhibits also the growth of the wild type ML3, will be dealt with in a forthcoming publication.

Table 1. *Competitive inhibition of the growth of the leucine-requiring mutant ML328c of* Esch. coli *by* L-*isoleucine*

(Incubation: 18 hr. In this and subsequent tables, the concentrations given are the final molar concentrations, and growth is expressed in units of the Meunier photometer. Inhibition index is defined below.)

L-Leucine (M)	L-Isoleucine (M)	Growth (units)	Inhibition index
5×10^{-6}	0	40	
5×10^{-6}	10^{-4}	16	
5×10^{-6}	$2{\cdot}5 \times 10^{-4}$	4	50
10^{-5}	0	97	
10^{-5}	10^{-4}	110	
10^{-5}	$2{\cdot}5 \times 10^{-4}$	49	
10^{-5}	5×10^{-4}	15	
10^{-5}	10^{-3}	1	50–100
$2{\cdot}5 \times 10^{-5}$	0	133	
$2{\cdot}5 \times 10^{-5}$	5×10^{-4}	122	
$2{\cdot}5 \times 10^{-5}$	10^{-3}	83	
$2{\cdot}5 \times 10^{-5}$	$2{\cdot}5 \times 10^{-3}$	11	
$2{\cdot}5 \times 10^{-5}$	5×10^{-3}	2	100–200
5×10^{-5}	0	135	
5×10^{-5}	10^{-3}	128	
5×10^{-5}	$2{\cdot}5 \times 10^{-3}$	127	
5×10^{-5}	5×10^{-3}	60	
5×10^{-5}	9×10^{-3}	1	180
10^{-4}	0	133	
10^{-4}	5×10^{-3}	143	
10^{-4}	9×10^{-3}	124	
10^{-4}	$1{\cdot}6 \times 10^{-2}$	18	approx. 160

The low inhibition index of L-isoleucine (that is the ratio L-isoleucine/L-leucine necessary to abolish totally the growth) rendered it feasible to study the inhibition by this amino-acid in more detail. The inhibition was found to be competitive. The inhibition index varies from 40 to 180 for L-leucine concentrations varying from $2{\cdot}5 \times 10^{-6}$ to 10^{-4}M (Table 1). This suggests that L-isoleucine interferes with L-leucine utilization by the mutant. It will become apparent that the inhibitory action of L-valine concerns also L-leucine utilization, although no evidence was obtained as to the competitive nature of the inhibition. The inhibitory effect on growth is restricted to the L-isomers of isoleucine and valine: D-isoleucine (with an index as high as 2000) and D-valine did not prove inhibitory.

Suppression by leucine peptides of the inhibition of the leucine-requiring mutant caused by L-isoleucine and L-valine

In the case here described, the inhibition by L-isoleucine of the growth of the leucine-requiring mutant ML328c on L-leucine could not be prevented by the addition to the medium of the following amino-acids or related compounds at the concentrations of 10^{-4} and 10^{-3}M: L-valine, DL-methionine,

Table 2. *Non-competitive suppression by* L-*leucylglycine of the inhibition of growth caused by* L-*isoleucine*

(Incubation: 20 hr. L-leucine: 10^{-5}M in all cases.)

L-Isoleucine (M)	L-Leucylglycine (M)	Growth (units)
0	0	85
10^{-3}	0	1
10^{-3}	5×10^{-6}	70
10^{-3}	10^{-5}	82
$2{\cdot}5 \times 10^{-3}$	0	0
$2{\cdot}5 \times 10^{-3}$	5×10^{-6}	55
$2{\cdot}5 \times 10^{-3}$	10^{-5}	73
5×10^{-3}	0	0
5×10^{-3}	5×10^{-6}	39
5×10^{-3}	10^{-5}	60
5×10^{-3}	$2{\cdot}5 \times 10^{-5}$	88
10^{-2}	0	0
10^{-2}	5×10^{-6}	31
10^{-2}	10^{-5}	50
10^{-2}	$2{\cdot}5 \times 10^{-5}$	79
10^{-2}	5×10^{-5}	114
2×10^{-2}	0	0
2×10^{-2}	5×10^{-6}	29
2×10^{-2}	10^{-5}	42
2×10^{-2}	$2{\cdot}5 \times 10^{-5}$	69
2×10^{-2}	5×10^{-5}	96
2×10^{-2}	10^{-4}	121

DL-threonine, L-histidine, L-arginine, DL-serine, L-phenylalanine, L-alanine, DL-lysine, α-ketoglutarate. The following combinations also proved ineffective: valine plus methionine, valine plus threonine, methionine plus threonine, valine plus methionine plus threonine. We considered then the possibility that the inhibitory amino-acids acted on the synthesis of leucine peptides presumably normally formed from leucine in the course of protein synthesis by the organism under study. We found that L-leucylglycine non-competitively reversed the inhibition caused by L-isoleucine (Table 2). This result suggested that L-leucylglycine was the product of the inhibited reaction. However,

Table 3. *Non-competitive suppression by glycyl-L-leucine of the inhibition of growth caused by L-isoleucine*

(Incubation: 22 hr. L-Leucine: 10^{-5} M in all cases.)

L-Isoleucine (M)	Glycyl-L-leucine (M)	Growth (units)
0	0	104
10^{-3}	0	7
10^{-3}	5×10^{-6}	101
10^{-3}	10^{-5}	114
$2{\cdot}5 \times 10^{-3}$	0	0
$2{\cdot}5 \times 10^{-3}$	5×10^{-6}	70
$2{\cdot}5 \times 10^{-3}$	10^{-5}	99
5×10^{-3}	0	4
5×10^{-3}	5×10^{-6}	57
5×10^{-3}	10^{-5}	81
5×10^{-3}	$2{\cdot}5 \times 10^{-5}$	118
10^{-2}	0	3
10^{-2}	5×10^{-6}	34
10^{-2}	10^{-5}	56
10^{-2}	$2{\cdot}5 \times 10^{-5}$	103
10^{-2}	5×10^{-5}	116
2×10^{-2}	0	3
2×10^{-2}	5×10^{-6}	31
2×10^{-2}	10^{-5}	41
2×10^{-2}	$2{\cdot}5 \times 10^{-5}$	82
2×10^{-2}	5×10^{-5}	106
2×10^{-2}	10^{-4}	127

glycyl-L-leucine appeared as active as L-leucylglycine in non-competitively suppressing the inhibition (Table 3). The two peptides suppress also the inhibition caused by valine (Table 4). Irregular results were obtained with the methionine inhibition.

Utilization of leucine peptides by the leucine-requiring mutant

If leucine peptides are the products of the reaction blocked by L-isoleucine, growth on these peptides instead of L-leucine should be insensitive to the presence of L-isoleucine, or at least the molar ratio of L-isoleucine to the peptide necessary to suppress the growth should be much higher than that of L-isoleucine to L-leucine. It was found: (*a*) that the growth response of the organism to both peptides is similar to that obtained on equimolar amounts of L-leucine or of L-leucine plus glycine; DL-leucylglycylglycine (calculated as the L-isomer) is as active for growth as L-leucylglycine; (*b*) that the growth of the organism on optimal concentrations of the peptides is completely insensitive to L-isoleucine and L-valine (Tables 5 and 6). This result seems to substantiate the hypothesis that the peptides are the product of the inhibited reaction.

Table 4. *Suppression by L-leucylglycine and glycyl-L-leucine of the inhibition of growth caused by valine*

(Incubation: 24 hr.)

L-Leucine (M)	L-Valine (M)	L-Leucylglycine (M)	Glycyl-L-leucine (M)	Growth (units)
10^{-5}	0	0	0	92
10^{-5}	10^{-2}	0	0	3
2×10^{-5}	10^{-2}	0	0	4
10^{-5}	10^{-2}	10^{-5}	0	93
10^{-5}	10^{-2}	0	10^{-5}	84

Table 5. *Insensitivity of the growth on leucine peptides to isoleucine*

(Incubation: 20 hr.)

L-Leucine (M)	L-Leucylglycine (M)	Glycyl-L-leucine (M)	L-Isoleucine (M)	Growth (units)
$2{\cdot}5 \times 10^{-5}$	0	0	0	80
$2{\cdot}5 \times 10^{-5}$	0	0	5×10^{-3}	1
0	$2{\cdot}5 \times 10^{-5}$	0	0	97
0	0	$2{\cdot}5 \times 10^{-5}$	0	87
0	$2{\cdot}5 \times 10^{-5}$	0	10^{-2}	73
0	$2{\cdot}5 \times 10^{-5}$	0	2×10^{-2}	63
0	0	$2{\cdot}5 \times 10^{-5}$	10^{-2}	71
0	0	$2{\cdot}5 \times 10^{-5}$	2×10^{-2}	55

Table 6. *Insensitivity of the growth on leucine peptides to valine*

(Incubation: 24 hr.)

L-Leucine (M)	L-Leucylglycine (M)	Glycyl-L-leucine (M)	L-Valine (M)	Growth (units)
10^{-5}	0	0	0	92
10^{-5}	0	0	10^{-2}	3
0	10^{-5}	0	0	100
0	0	10^{-5}	0	95
0	10^{-5}	0	2×10^{-2}	81
0	0	10^{-5}	2×10^{-2}	90

Hydrolysis of leucine peptides by washed suspensions of the leucine-requiring mutant

To interpret our results, it was important to ascertain whether or not the peptides were hydrolysed by the leucine mutant. We used the technique of paper chromatography to test this possibility.

Esch. coli ML328*c* (1 l.) was grown for 18 hr. in a synthetic medium containing L-leucine (2×10^{-5} M). The culture was then centrifuged, the cells washed twice with saline and resuspended in 3 ml. of sterile water. Four tubes were run for each peptide, and contained 5 mg. of peptide, 0·12 ml. water and 0·13 ml. bacterial suspension (containing 2 mg. N/ml.). The total volume was 0·75 ml. Tubes were also run that contained bacteria plus leucine, bacteria plus glycine, bacteria plus both amino-acids and bacteria alone. The tubes were incubated at 37° for periods of 30 min., 1, 4 and 24 hr. After centrifugation, a sample of the supernatant was spotted on Whatman no. 1 filter paper and ascending chromatography was run with butanol-acetic acid as solvent. The chromatogram was developed with ninhydrin (0·1 %) in $CHCl_3$ containing 0·1 % collidine.

In the case of glycyl-L-leucine, no hydrolysis was evident after 0·5, 1 and 4 hr. After 24 hr. slight spots of glycine and leucine were present, which indicated a slight hydrolysis. In the case of L-leucylglycine, very slight hydrolysis was noticeable as early as 0·5 hr., and increased up to 24 hr.

Peptide specificity

DL-Alanylglycine, glycylglycine, glycylglycylglycine, glycyl-L-tyrosine, glycyl-L-tryptophan did not reverse the inhibition caused by L-isoleucine of the growth of the leucine-requiring mutant. Other peptides containing or not containing leucine were not available.

DISCUSSION

An explanation of the observed facts might be based on the now classical criteria concerning the interpretation of competitive inhibition. According to these concepts, the fact that supplying the organism with L-leucylglycine or glycyl-L-leucine suppresses the inhibition in a non-competitive manner, suggests that it is the synthesis of leucine peptides from L-leucine which is inhibited; moreover, the fact that these compounds meet the needs of the organism and allow growth independently of the amount of L-isoleucine (or L-valine) points to the same conclusion.

It is difficult to visualize the activity of leucine peptides in suppressing the inhibition caused by isoleucine and valine as resulting from a mere hydrolysis of the peptides to free leucine. In this case, there would still be a competitive inhibition by the inhibitors of the growth on the peptides. Another alternative would be the incorporation, as such, of the peptides in the bacterial protein. This is rendered improbable by the fact that both L-leucylglycine and glycyl-L-leucine are equally active for growth. It would therefore be difficult to account for the fact that either of the two types of preformed linkage is sufficient to overcome the inhibition caused by isoleucine and valine and to ensure alone the growth of the organism.

Hydrolysis of the peptides could give rise to a form of L-leucine more active for growth, i.e. more readily converted to other peptides. For example, transpeptidation reactions could occur, the supplied preformed peptide being capable of transpeptidation with other amino-acids or other peptides to give all the leucine peptide configurations necessary for the synthesis of bacterial protein. Such reactions have been shown to occur in kidney and pancreas extracts between glutathione on the one hand and leucine, phenylalanine or valine on the other (Hanes, Hird & Isherwood, 1950). The products of the reaction were cysteinylglycine and three new substances. In the case of phenylalanine, the substance has been identified on reasonably good grounds with γ-glutamylphenylalanine. There is good reason to support the suggestion that the proteolytic enzymes themselves may catalyse transpeptidation (Lipmann, 1949; Frantz & Lotfield, 1950; Brenner, Müller & Pfister, 1950; Fruton, 1950). The existence in the leucine-requiring mutant ML328*c* of peptidases that hydrolyse the peptides used in this study is thus not incompatible with the hypothesis of a utilization of the peptides other than through mere hydrolysis. Hanes *et al.* (1950) announce that they recently obtained with plant enzymes more direct evidence of transpeptidation reactions involving, this time, α-peptides.

Transpeptidation reactions would certainly account for the equal activity of the two different peptides of leucine tested in the present study, as well as for the results of Simmonds & Fruton (1948) with their proline-requiring mutants. It appears reasonable to us to consider this possibility as a likely one.

Further work is being done in this laboratory to obtain more information on possible transpeptidation reactions and on their possible significance for the synthesis of bacterial protein.

SUMMARY

1. The growth of a leucine-requiring mutant of *Escherichia coli* is inhibited by the presence in the culture medium of L-isoleucine, L-valine, DL-methionine and DL-norleucine. D-Valine and D-isoleucine are not inhibitory.

2. The inhibition by L-isoleucine is competitive.

3. The inhibition of the growth of the mutant caused by L-isoleucine and L-valine is suppressed in a non-competitive way by the addition of either L-leucylglycine or glycyl-L-leucine.

4. Different explanations for the results obtained are considered, taking into account that the presence in the mutant of the peptidases that hydrolyse the peptides studied is not incompatible with a utilization of the peptides prior to their mere hydrolysis, for example, through transpeptidation reactions.

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Acetylcholine

1. HYDROLYSIS BY HYDROGEN AND HYDROXYL ION

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(*Received* 22 *November* 1951) .

This paper presents a study of the acid- and alkali-catalysed hydrolyses of acetylcholine bromide, which may be written

(1) $CH_3COOCH_2CH_2N^+(CH_3)_3 + H_2O + H_3O^+ \rightleftharpoons CH_3COOH + HOCH_2CH_2N^+(CH_3)_3 + H_3O^+$,

(2) $CH_3COOCH_2CH_2N^+(CH_3)_3 + OH^- \rightleftharpoons CH_3COO^- + HOCH_2CH_2N^+(CH_3)_3$.

Our object has been to obtain kinetic data leading to frequency factors and activation energies, for comparison with similar data on the neutral hydrolysis catalysed by true and pseudo cholinesterases. A comparison of the data obtained here with published data on non-ionic esters, such as methyl acetate, has an intrinsic theoretical interest for reaction mechanisms, because of the positive charge on the acetylcholine ion. To date, the study of the non-enzymic hydrolysis of acetylcholine has been limited to obtaining the percentage non-enzymic hydrolysis in 20 min. at pH values near to 7 (Easson & Steadman, 1936). We shall discuss the equilibrium point of the reaction in a later paper, but in both cases it is far enough to the right for us to neglect the back reaction in our kinetic analysis.

EXPERIMENTAL AND RESULTS

The hydrogen-ion catalysis

Method. Commercial acetylcholine bromide was recrystallized from absolute ethanol until it reached a constant melting point of 143°. Conventional methods were used to follow the reaction. Hydrochloric acid catalyst solution (100 ml.) was brought to the thermostat temperature in a stoppered conical flask. Four concentrations of acid were used, 0·1, 0·075, 0·05 and 0·025 N-HCl. At zero time, a weighed amount of acetylcholine bromide was added and the flask shaken, so that the bromide rapidly dissolved. After varying time intervals (decided after preliminary experiments), 5 ml. portions of the reaction mixture were withdrawn and run into a known amount of standardized NaOH solution plus phenolphthalein, in a conical flask at 0°. The NaOH solution was calculated to neutralize the catalyst present. The solution was then rapidly titrated to the end point with further NaOH. If the titre at infinite time is r_∞ and at time t is r_t, the quantity of ester remaining unhydrolysed at time t is equivalent to $r_\infty - r_t$. The difficulties associated with the determination of r_∞ were avoided by the use of Guggenheim's (1926) method. If r_t be the titration value at time t and $r_{t+\tau}$ the titration value at time $t+\tau$, where τ is a suitable standard time interval, chosen to be at least twice the half-life of the reaction, then Guggenheim's method gives the first-order constant k as the slope of the

plot of $2{\cdot}303 \log_{10} (r_t - r_{t+\tau})$ against time t. A specimen plot in Fig. 1 shows the reaction is accurately first order in the ester concentration, as is the case for non-ionic esters. The first-order constants are given in Table 1.

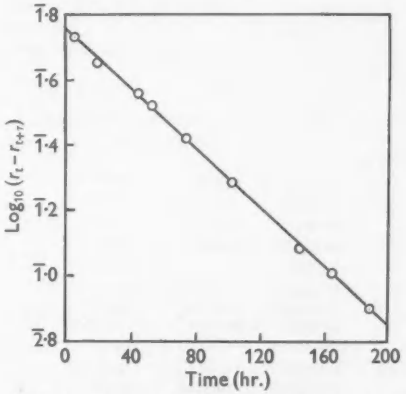


Fig. 1. First-order plot for 2·2718 g. acetylcholine bromide in 100 ml. 0·1 N-HCl at 25°.

Table 1

(*a*) *First-order constants, k min.*$^{-1} \times 10^3$, *for the hydrolysis of acetylcholine*

HCl (N)	t (°) 25	37	46·1	60
0·1	0·181	0·603	1·82	3·61
	0·182	0·552	1·70	3·52
0·075	0·142	0·421	1·47	—
	0·134	0·442	1·63	2·65
0·05	0·0833	0·241	0·668	1·68
	0·081	0·258	—	1·41
0·025	0·035	0·108	—	0·783
	0·0391	—	—	—

(*b*) *The catalytic constant* k_{H^+} *l.mole*$^{-1}$ *sec.*$^{-1} \times 10^5$, *for the reaction*

t (°)	...	25	37	46·1	60
k_{H^+}	...	3	9·7	30	51·7

Results. If the reaction is first order in the concentration of hydrochloric acid catalyst, then the catalytic constant k_{H^+}, the second-order constant, is given by $k = k_{H^+}[H_3O^+]$, where $[H_3O^+]$ is the concentration of hydrochloric acid. Fig. 2 shows this to be so, within the experimental accuracy. Values of k_{H^+} are given in the last line of Table 1, their accuracy being about ± 5 %, with the exception of the point at 46·1°, which is less accurate for some reason.

Experiments were carried out at 25, 37, 46·1 and 60°. In Fig. 3, the results are plotted as $\log_{10} k_{H^+}$ against $1/T$. The line has been drawn according to the method of least squares, and the activation energy corresponding to the slope of this line is 16 570 cal./mole, with a probable error of 1522 cal./mole.

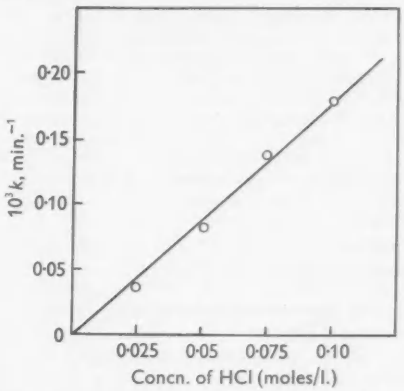


Fig. 2. Evaluation of k_{H^+}, the second-order velocity constant at 25°.

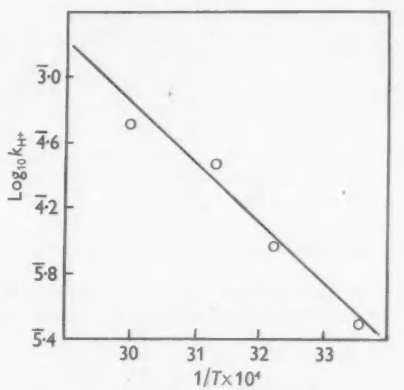


Fig. 3. Arrhenius plot for the hydrogen-ion-catalysed hydrolysis.

The Arrhenius equation is $k_{H^+} = Ae^{-E/RT}$, and from the intercept in Fig. 3, we obtain

$$A = 4{\cdot}7 + 10^7 \text{ l.mole}^{-1}\text{sec.}^{-1},$$

with a probable error of a factor of 10.

The hydroxyl-ion catalysis

Method. Although this reaction was rather rapid, it was found possible to follow it by the normal titration method. For a given experiment, 25 ml. of standardized NaOH solution were brought to the required temperature in a thermostat, together with a flask containing the acetylcholine solution. To start the reaction, 10 ml. of the acetylcholine were pipetted into the NaOH, the stop-watch being

started when half the solution had been transferred. The solution was vigorously shaken for a few seconds and after lapse of the appropriate time intervals, stopped by adding an excess of standardized HCl solution. The amount of alkali used up in the saponification was then determined by titration of the solution against standard NaOH solution, using phenolphthalein, until the first sign of a pink coloration (this, of course, rapidly disappeared as the small excess of alkali induced further saponification). Six experiments were made for each complete run, at a series of time intervals up to 120 sec., for temperatures 0, 10, 12·5 and 15°, and up to 20 sec. for 18 and 20°. The rather rapid technique necessary was secured by the co-operation of two workers. From the titration data we obtain a = initial concentration of NaOH; b = initial concentration of acetylcholine bromide; x = concentration of product at time t.

Results. The data fit the second-order law, examples being given in Fig. 4.

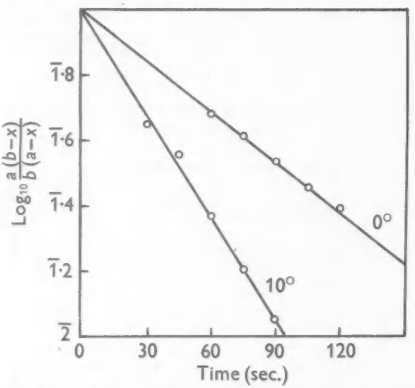


Fig. 4. Saponification of acetylcholine bromide at 0 and 10°.

Two or more runs were done at each temperature, and second-order constants k_{OH^-} calculated as below are given in Table 2.

$$k_{OH^-} = \frac{2{\cdot}303}{t(b-a)} \log_{10} \frac{a(b-x)}{b(a-x)}.$$

To obtain the activation energy E, we have plotted average values of k_{OH^-} as $\log_{10} k_{OH^-}$ against $1/T°K$ in Fig. 5. The result is $E = 12\,180$ cal./mole, with a probable error of 240 cal./mole.

The equation for the absolute rate is obtained as

$$k_{OH^-} = 1{\cdot}0 \times 10^9 \, e^{-12\,180/RT} \text{ l.mole}^{-1}\text{sec.}^{-1}.$$

The probable error on the A factor is a factor of 3·8.

DISCUSSION

In Table 3 we compare our data with the similar data on the hydrolysis of the non-ionic esters. It is apparent that the hydrogen-ion catalysis has a closely similar activation energy for acetylcholine, methyl acetate and ethyl acetate. The acetylcholine hydrolysis goes more slowly, the difference residing apparently in a smaller A factor. To discuss the matter further would require a critical discussion of the errors involved in the determination of E in the investigation concerned. It is interesting that 60 % aqueous acetone behaves very similarly to water as a solvent in this reaction.

Table 2. *Second-order velocity constants,* k_{OH^-}, *l.mole*$^{-1}$*sec.*$^{-1}$, *for the alkaline hydrolysis of acetylcholine*

Temp. (°)	Initial [AChBr] (mole/l.)	Initial [NaOH] (mole/l.)	$k_{OH^-} \times 10^1$	$k_{OH^-} \times 10^1$ (average)
0	0·046366	0·10413	2·044	2·018
	0·061913	0·09017	1·992	
10	0·034068	0·089942	4·294	4·573
	0·036967	0·089942	4·262	
	0·03781	0·03781	4·861	
	0·035375	0·036861	4·874	
12·5	0·04882	0·069776	5·493	5·375
	0·052396	0·063798	5·257	
15	0·049613	0·065283	6·860	6·588
	0·038717	0·061502	6·316	
18	0·03624	0·085547	8·406	8·175
	0·044136	0·085547	7·943	
20	0·042776	0·071285	9·491	9·506
	0·046005	0·071285	9·521	

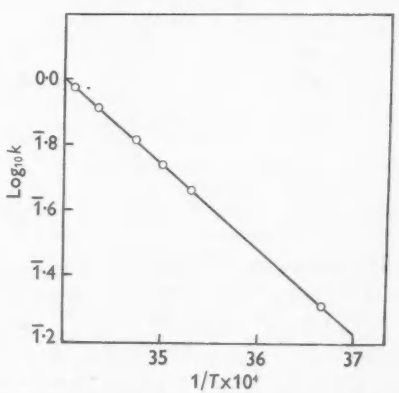


Fig. 5. Arrhenius plot for the hydroxyl-ion hydrolysis.

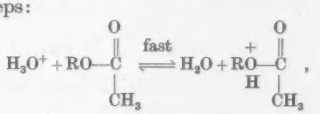
In the case of the hydroxyl-ion reaction, the acetylcholine reaction at 0° has a value of k_{OH^-} about 10 times that for ethyl acetate and methyl acetate, and the results show rather definitely that this is due to a larger A factor.

The hydrolysis of esters is supposed to take place in consecutive steps, the basic ideas being formulated by Lowry (1925, 1927), and recent developments discussed by Watson (1941) and Day & Ingold (1941). In the form set forth by the latter

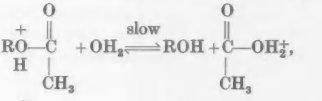
Table 3. *Comparison of kinetic data for ester hydrolysis*

Ester	Catalyst	Solvent	k (l.mole^{-1} sec.$^{-1}$)	Reference
Acetylcholine	H_3O^+	H_2O	$4{\cdot}7 \times 10^7\ e^{-16\,570/RT}$	Present paper
Methyl acetate	H_3O^+	H_2O	$3{\cdot}86 \times 10^8\ e^{-16\,920/RT}$	Lamble & Lewis (1914) Moelwyn-Hughes (1947)
Ethyl acetate	H_3O^+	H_2O	$1{\cdot}66 \times 10^8\ e^{-16\,830/RT}$	Taylor (1915) Moelwyn-Hughes (1947)
Methyl acetate	H_3O^+	60% (v/v) aq. acetone	$4{\cdot}36 \times 10^7\ e^{-16\,250/RT}$	Newling & Hinshelwood (1936)
Acetylcholine	OH^-	H_2O	$1{\cdot}0 \times 10^9\ e^{-12\,180/RT}$	Present paper
Ethyl acetate	OH^-	H_2O	$3{\cdot}79 \times 10^7\ e^{-11\,660/RT}$	Potts & Amis (1949)
Methyl acetate	OH^-	60% aq. acetone	$3{\cdot}7 \times 10^7\ e^{-11\,500/RT}$	Newling & Hinshelwood (1936)

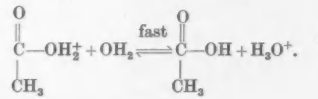
authors, the hydrogen-ion catalysis goes through three steps:



(1)



(2)

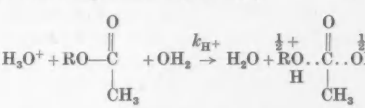


(3)

The observed velocity constant k_{H^+} will then be K_1k_2 where K_1 is the equilibrium constant for reaction (1), that is, k_1/k_{-1} where k_1 and k_{-1} are the rate constants of forward and reverse steps (1), and k_2 is the rate constant of forward step (2), which determines the rate. For consideration of the energy change ΔH^*, and the entropy change ΔS^* in this process (Glasstone, Laidler & Eyring, 1941), as defined by

$$\Delta H^* = E - RT, \quad A = \frac{kT}{h}\, e^{\Delta S^*/R},$$

where E is the experimental activation energy, we may put steps (1) and (2) together and consider the changes in going from the three reactants to the activated complex of reaction (2)



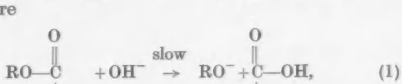
The evidence is that on changing from R as choline with a positive charge to R as methyl or ethyl with zero charge, that the rate of this process k_{H^+} is slightly increased. This is qualitatively what we might expect on the basis of electrostatic charges repelling each other.

Christiansen (1924) inserted a term for the electrostatic energy of two reacting ions into the expression for the activation free energy and Moelwyn-Hughes (1936, 1947) has given formulae for A which show that we shall expect a lowering of A in this case below that for the ion-neutral molecule reaction. Glasstone *et al.* (1941) have given the equivalent formulation of ΔS^* in terms of activated complex theory. The latter formulation is

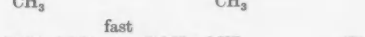
$$\delta\Delta S^* = -10 z_a z_b \text{ cal. deg.}^{-1} \text{ mole}^{-1}$$

for the difference between the ion-ion and ion-neutral molecule reactions. Since $z_a z_b = 1$, we expect a lowering of the A factor by e^{-5}, i.e. 10^{-2}, but there are reasons for believing this term may be nearer unity when the ionic charges may resonate over several atoms as in the present case.

The steps suggested for the hydroxyl-ion catalysis are

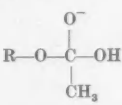


(1)

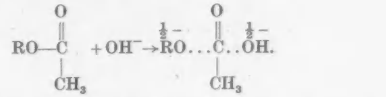


(2)

where (1) may be split into an initial rapid reversible formation of the species



and its slow decomposition. In any event, the relevant change is from the primary reactants to the activated complex below



Exactly the same argument applies as above, only, since $z_a = 1$ and $z_b = -1$, we shall expect an increase in A factor of about 10^2 as an upper value.

Thus, we conclude that the rather small differences evidenced in the hydrolysis of acetylcholine and a non-ionic ester such as methyl acetate fit in with what we know about the slow steps and activated complexes in these reactions.

SUMMARY

1. The kinetics of hydrolysis of acetylcholine bromide by hydrogen ion and hydroxyl ion have been followed by conventional methods. The bimolecular velocity constants respectively are, in l.mole^{-1}sec.$^{-1}$,

$$k_{H^+} = 4{\cdot}7 \times 10^7 \ e^{-16\,570/RT}$$

and $$k_{OH^-} = 1{\cdot}0 \times 10^9 \ e^{-12\,180/RT}.$$

2. These data are very similar to those for non-ionic esters, such as methyl acetate. The A factor for k_{H^+} is about 5 times smaller than for such esters, while for k_{OH^-} it is about 20 times larger. These differences find an explanation in the ionic charge of the activated complex.

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Acetylcholine

2. THE HEAT OF HYDROLYSIS

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A recent hypothesis of nerve conduction (Nachmansohn, 1945, 1950) supposes that the depolarization of the nerve membrane is brought about by acetylcholine, and that the polarized state is renewed by the hydrolysis of the acetylcholine by its esterase. The synthesis of acetylcholine is supposed to involve the high-energy phosphate bond in acetyl phosphate, in turn synthesized from creatine phosphate. In support of these views, there is the evidence that in *Electrophorus electricus* the energy release calculated for the observed creatine phosphate hydrolysis approximately equals the energy of electrical discharge. Nachmansohn has, however, pointed to a discrepancy in the energy balance of the postulated recovery process. Meyerhof & Schulz (1935) found $\Delta H_1^0 = -10\,700$ cal./mole for the neutral hydrolysis of creatine phosphate, from which Lipmann (1941) has calculated $\Delta G_1^0 = -10\,000$ cal. Lipmann (1946) subsequently corrected his calculated value for the free energy of hydrolysis of phosphopyruvate by 4700 cal. and since his creatine phosphate figure was based on the phosphopyruvate figure, it is appropriate to apply this correction to creatine phosphate, which now brings its free energy of hydrolysis to $-14\,700$ cal. Within the accuracy of the calculations this may be taken as 15000 cal. If the ΔG_1^0 for acetylcholine synthesis is similar to the values for other esters, it will be close to zero cal. and there will, therefore, be a loss of 15000 cal./mole in the recovery process. As a first step in an investigation of ΔG_1^0, we have measured the heat of alkaline hydrolysis $-\Delta H_2^0$, from which we can calculate the value for hydrolysis by neutral molecules $-\Delta H_1^0$, and thus obtain an estimate of ΔG_1^0. Later we hope to obtain the equilibrium constant K, and thus $\Delta G_1^0 = -RT \ln K$, using hydrogen ion as a catalyst. Since this work was completed, Hestrin (1950) has published a value for K and ΔG_1^0, using the enzyme-catalysed reaction. We shall consider this work in the discussion.

Thermochemistry of the hydrolysis

The hydrolysis of acetylcholine by cholinesterase at physiological pH may be written:

$$CH_3COOCH_2CH_2N^+(CH_3)_3 + 2H_2O \rightarrow CH_3COO^- + H_3O^+ + HOCH_2CH_2N^+(CH_3)_3 \quad (\Delta H_1^0)$$

The enzyme-catalysed reaction, with the enzyme preparations available to us, was rather too slow for the vacuum-jacket calorimeter used in this paper. We have accordingly measured the heat of hydrolysis by alkali,

$$CH_3COOCH_2CH_2N^+(CH_3)_3 + OH^- \rightarrow CH_3COO^- + HOCH_2CH_2N^+(CH_3)_3 (\Delta H_2^0).$$

If we have the quantity ΔH_3^0 defined by the equation

$$2H_2O \rightarrow H_3O^+ + OH^- \quad \Delta H_3^0 = 13\,490 \text{ cal.},$$

then

$$\Delta H_1^0 = \Delta H_2^0 + \Delta H_3^0,$$
$$= \Delta H_2^0 + 13\,490 \text{ cal.}$$

The ΔH_3^0 value is taken from the compilation of Everett & Wynne-Jones (1939).

Since choline is a strong base, no correction is necessary for the formation of undissociated $HOCH_2CH_2N(CH_3)_3OH$. The dissociation constant for choline, reported by Price & Lewis (1933) as $8{\cdot}71 \times 10^{-6}$ at room temperature, has been found to be in error, in agreement with the general rule that all quaternary nitrogen hydroxides are completely dissociated.

EXPERIMENTAL

Method. The calorimeter used has been described in another paper (Dilke, Eley & Sheppard, 1950). A Dewar vessel was used, temperature being measured by the resistance change in a thermistor, and the water equivalent obtained by an electrical resistance coil. A quantity of acetylcholine was weighed out and dissolved in 60 ml. water in the calorimeter. The delivery pipette contained 5 ml. of 1·3 N-NaOH, which always sufficed to give an excess of alkali over acetylcholine. The calorimeter was immersed in a 25° thermostat, and when temperature equilibrium of the contents had been obtained the NaOH was blown over from the pipette into the acetylcholine and the resistance-time record taken with the thermistor. Under these conditions, the hydrolysis reaction went virtually to completion. Finally, the water equivalent was determined. The cooling corrections were made by White's method as outlined in the paper quoted.

The procedure was to hydrolyse different weights of acetylcholine w, so as to give heats varying from 5 to 35 cal., which could be measured to an accuracy of 0·1 cal. If M is the molecular weight of acetylcholine, Q its heat of hydrolysis per mole (i.e. $-\Delta H_2^0$), then the actual heat x evolved in any experiment will be

$$x = D + (Q/M)\, w,$$

where D should be the heat of dilution of the NaOH solution in 60 ml. water. Thus, from the slope of the straight line connecting x and w, we determine $Q = -\Delta H_2^0$.

Materials. Acetylcholine bromide. The B.D.H. (British Drug Houses Ltd.) product recrystallized from ethanol as colourless needles, m.p. 142–143°.

Acetyl-β-methylcholine chloride. The Savory and Moore product (Amechol) was washed with ether, m.p. 172°.

Benzoylcholine chloride. This was prepared from β-chloroethyl benzoate and trimethylamine by the method recommended by Easson & Steadman (1936). The yield was 50%, m.p. 204°.

Choline bromide. The B.D.H. product was recrystallized from ethanol.

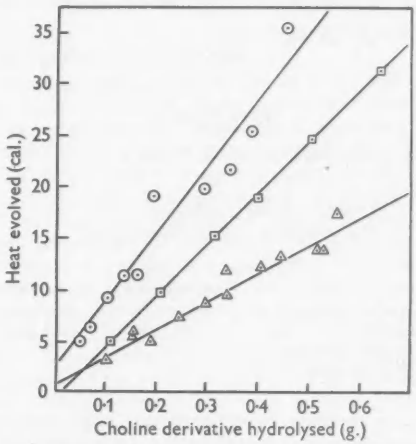

Fig. 1. Heat evolved, as a function of weight of choline derivative hydrolysed. ⊙, acetyl-β-methylcholine chloride; ⊡, benzoylcholine chloride; △, acetylcholine bromide.

RESULTS

The heat of dilution, D, was found to be negligible in a separate experiment. The results of experiments with acetylcholine bromide, Amechol and benzoylcholine chloride are shown in Fig. 1. In each case the best straight line has been drawn through the points by the method of least squares, and the probable error has been calculated for the value of the heat Q. In the case of benzoylcholine chloride the points lie very well on a straight line and the error is less than 0·1 k.cal./mole and has been omitted. In the case of choline bromide the heat of mixing with sodium hydroxide was found to be negligible.

Hydrolysis of choline chloride. A salt of a strong acid with a weak base (basic constant K_b) gives an acid solution. If C be the concentration of salt, K_w the dissociation constant of water, a measurement of the pH will give a value of K_b, using the equation

$$pK_b = pK_w - \log C - 2pH.$$

Thus, Price & Lewis (1933) found a choline chloride solution of $C = 0{\cdot}038$ mole/l. to have a pH of 5·13, leading to $pK_b = 5{\cdot}16$. These results were obtained with both gold-quinhydrone and glass electrodes, and it is not easy to see where the error lies. In our case, we found the initial pH to be quite

near to neutrality, 0·043 N solution having a pH of 6·88. However, there is a difficulty in that the pH of choline chloride solutions from which carbon dioxide is excluded tends to drift down to 5·7. A hydrolysis reaction may be responsible, as we have noticed trimethylamine on exposing wet crystals to the air, thus possibly

$$HOCH_2CH_2NMe_2Cl + H_2O = HOCH_2CH_2OH + NMe_3 + HCl.$$

DISCUSSION

The results are collected in Table 1.

Table 1. *Heats of alkaline* ($-\Delta H_2^0$) *and neutral* ($-\Delta H_1^0$) *hydrolysis*

Ester	$-\Delta H_2^0$ (kg.cal./mole)	$+\Delta H_1^0$ (kg.cal./mole)
Acetylcholine bromide	12·4±0·2	1·09±0·2
Amechol	12·8±0·7	0·69±0·7
Benzoylcholine bromide	12·2	1·29
Choline bromide	0·0	—

The decrease in experimental error over the series Amechol, acetylcholine, benzoylcholine is noteworthy. This order is, in fact, the order of decreasing hygroscopicity, so the error probably arises in weighing out the substances for hydrolysis. In all cases, the time of reaction is 1–1·5 min., and so the error cannot arise from differences in cooling corrections. It is hoped at some later date to obtain values of ΔH_1^0 from enzyme-catalysed hydrolyses in an adiabatic calorimeter. Both the heat of mixing of choline bromide and sodium hydroxide and the pH measurements on choline chloride solution agree in classifying choline as a strong base, so no correction to ΔH_2^0 is necessary for incomplete dissociation of the choline hydroxide.

The Gibbs free energy change ΔG_1^0 (Hestrin writes it ΔF) for neutral hydrolysis is given by

$$\Delta G_1^0 = -RT \ln K,$$

where $K = \dfrac{[CH_3COOH]\,[ROH]}{[CH_3COOR]\,[H_2O]}$. Since Hestrin (1950) finds $K = 4$ at $T =$ about 296°, $\Delta G_1^0 = -820$ cal./mole, and hence the entropy change ΔS_1^0 is

$$\Delta S_1^0 = \frac{\Delta H_1^0 - \Delta G_1^0}{T} = 6{\cdot}4 \text{ cal./deg.mole.}$$

This is a not unreasonable value. The value of -820 cal./mole for ΔG_1^0 corresponds to the same standard state for all reactants, e.g. 1 molal or 1 molar. The actual figure given by Hestrin is for a standard state for the water (55·5 M), and for the other reactants at 1 M concentration.

The neutral hydrolysis of ethyl acetate, classically investigated by Berthelot and St Gilles, does not go as far as that of acetylcholine. Thus, for this case, $K = 0{\cdot}25$ (cf. Parks & Huffman, 1932) and thus $\Delta G_1^0 = 820$ cal./mole. The heat of this hydrolysis does not seem to have been measured, so that we cannot calculate ΔS_1^0 for comparison with the above value. The error involved in heats of combustion is too large to make it worth while to use these data to calculate ΔH_1^0 for comparison with acetylcholine.

To summarize, with a reasonable value for the entropy of hydrolysis, our values of ΔH_1^0 will lead to a value for ΔG_1^0, the free energy change of hydrolysis, in the neighbourhood of zero, in agreement with the value recently published by Hestrin (1950). This work thus confirms the suggestion made by Nachmansohn (1945) that a synthesis of acetylcholine, starting with creatine phosphate, will involve a free energy loss of about 14000 cal./mole.

We hope to give further data on the free energy and entropy of the acetylcholine hydrolysis when we have completed our investigations of the equilibrium constant of the acid-catalysed reaction.

SUMMARY

1. Values of ΔH_{298}^0 have been measured for the alkaline hydrolysis of acetylcholine bromide and related substances. These lead to small values for the neutral hydrolysis, similar to those found for ordinary esters, in contrast to the high values characteristic of creatine phosphate hydrolysis.

2. There will be a loss of free energy of about 14000 cal. in a postulated synthesis of acetylcholine starting from creatine phosphate.

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Observations on the Composition and Activity of Partially Arylated Insulin

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One result of the crystallization of insulin and its identification as a protein hormone has been the stimulation of considerable research into the nature of its action and inquiry into the reasons why this particular molecule should be endowed with its unique physiological properties. It has been suggested as the result of experiments by Fraenkel-Conrat & Fraenkel-Conrat (1950) that although some amino-acid side chains must remain intact if full hormonal activity is to be retained, others may be modified without its destruction. Included in the latter category are the free terminal amino groups and the ϵ-amino groups of lysine. There are, however, certain points of criticism that may be raised against these findings.

In the first place, the amino groups in these experiments were masked by acetylation and, in view of the dynamic state of the proteins of the living organism, it is by no means certain that the acetyl residue could not be removed *in vivo*. Secondly, differently placed groups may be essential in different degrees, which would have the result that inhibition of activity might be a function of the size of the masking group. It is interesting to note in this connexion that Fraenkel-Conrat & Fraenkel-Conrat (1950) report some destruction of activity after coupling with phenyl isocyanate. This was attributed, however, to reaction with some of the essential tyrosine residues.

The suggested non-essential nature of the free amino groups of lysine leads to a third query based on the recent work of Lens (1949), who reported that digestion of crystalline insulin with carboxypeptidase resulted in the removal of about two terminal alanine residues per molecule, leaving a slightly modified insulin which was no longer active. Moreover, the work of Sanger & Tuppy (1951) has shown that the free carboxyl terminals of the pair of so-called *B* chains are derived from alanine residues and that the penultimate units of these chains are lysine. The whole of the lysine present in insulin is accounted for by these two residues. These two investigations seem to show that although the terminal alanine residue must be left intact in order to retain insulin activity, the immediately adjacent residues could be termed unessential. This situation was thought to be sufficiently curious to warrant further study.

In a search for a masking residue which was not only larger than acetyl, but which would form with an amino group a bond less likely to be broken by metabolic processes, a re-investigation has been made of the work of Barger & Tutin (1918). As originally used, this technique involved the reaction of 2:4:5-trinitrotoluene with the protein, when a molecule of nitrous acid was eliminated between any free amino group and a molecule of the reagent. On hydrolysis, coloured amino-acid derivatives analogous to those produced by the well-known method of Sanger (1945) could be isolated. An advantage of this reagent was that the reaction has been found to be slower than that with fluorodinitrobenzene and could be more easily stopped at any desired stage.

This paper describes the results of a preliminary re-examination of the essential nature of the amino groups of insulin and indicates the possible uses of active polynitro aromatic compounds in studies of this kind. Although 2:4:5-trinitrotoluene was the reagent finally selected, several other similar active nitro compounds were tried, specimens of which were liberally provided from the collection of Dr Edward Ward of Leicester Technical College, whose advice has greatly furthered these investigations.

METHODS

Formation of the polynitro derivative of insulin. About 100 mg. of crystalline insulin, prepared by Boots Pure Drug Co. Ltd., were dissolved in about 10 ml. of water to which a few drops of the required buffer had been added. Ethanol was then added to the clear liquid to a final concentration of 70%, followed by about 50 mg. of 2:4:5-trinitrotoluene (2:4:5-TNT). Buffer solution was added dropwise until the mixture was just saturated with salt. It was then allowed to stand with occasional shaking for various periods of time from 3 to 24 hr. at temperatures between 18 and 37°. After the required time, the mixture was diluted with water, the ethanol and excess reagent were extracted with ether and the pH of the aqueous layer adjusted to the isoelectric point. The resulting yellow precipitate was centrifuged off.

Buffer solutions. The pH of the reaction medium was maintained with Sørensen's phosphate buffers of pH 7·6, 7·8 or 8·3, or with dilute $NaHCO_3$ solution of pH 9·3. The actual pH values of these buffers was increased by solution in 70% ethanol however. The pH values of the ethanolic solutions saturated with buffer were estimated with a glass electrode to be 9·7, 9·2, 9·4 and 10·4 respectively, where the pH in

ethanol has been defined according to Michaelis & Mizutani (1925). It is not clear why the buffer of pH 7·6 gave so high a value when dissolved in the ethanolic medium.

Estimation of the nitro groups introduced. In early experiments this was done by reduction of the intact insulin derivative using $TiCl_3$. The blank determinations were rather large, however, so the method was discarded in favour of the analysis of the hydrolysis products of the arylated protein by the method of Mills (1952). Independent measurements on the synthetic dinitrotolyl (DNT) amino-acids showed that their breakdown during acid hydrolysis was not significantly different from that undergone by the corresponding dinitrophenyl derivatives (Porter & Sanger, 1948).

Assay of insulin activity. It was not possible to make very accurate assays as the appropriate facilities were not available. Consequently, the preparations described below were each tested in only two or three rabbits. The fall in blood sugar was measured at hourly intervals for 3 hr. after injection and compared with the values obtained by injection of standard samples of insulin. The concentration of protein in the solutions used was calculated from micro-Kjeldahl estimates of the total N.

RESULTS

It was necessary as a preliminary step to decide whether 2:4:5-TNT reacted appreciably with the phenolic groups of tyrosine. Preliminary qualitative experiments were first made with leucyltyrosine and acetyltyrosine ethyl ester. These substances were both allowed to react separately with 2:4:5-TNT in 70 % ethanol containing pH 7·8 buffer in one set of experiments and bicarbonate in the other. The DNT derivatives were hydrolysed in 6N-hydrochloric acid and examined on a paper chromatogram to discover whether any *O*-DNT-tyrosine had been formed. These tests showed that although little or no masking of tyrosine hydroxyl groups took place at the lower pH, some were destroyed in the more alkaline medium. The fact that some tyrosine remained unattacked even in the presence of bicarbonate was confirmed for the DNT-insulin by paper chromatography of hydrolysates.

In an attempt to obtain more information on this point, 2:4:5-TNT was allowed to react for 18 hr. with an equimolar amount of free tyrosine under the two sets of conditions already mentioned. The products were analysed chromatographically on a column of kieselguhr according to Mills (1952). In each experiment, two substances were found which were easily separated into yellow bands. The first of these ran at the speed of the diarylated tyrosine and gave no reaction with the Folin phenol reagent. The second band ran more slowly, as would be expected if it were the mono-substituted derivative, and gave a positive phenol reaction. It was concluded that the tyrosine amino group reacted more rapidly than the hydroxyl group, the reaction mixture therefore containing both di-DNT-tyrosine and *N*-DNT-tyrosine. The molecular ratio of mono-substituted tyrosine to di-substituted tyrosine, obtained at pH 10·4, was 1·34. At pH 9·2 the ratio was 13·1. In both experiments 70 % of the tyrosine remained unreacted when the experiment ended. It seemed probable from these experiments that while considerable substitution of the tyrosine residues of insulin was to be expected during reaction in the presence of bicarbonate, at pH 9·2 it was not likely to exceed about 0·5 residue/molecule.

These conclusions have been further examined by the reduction of intact DNT-insulin. If histidine or tyrosine had been masked, the total number of reducible groups found in this way would exceed the total found by the individual estimation of DNT-glycine, DNT-phenylalanine and DNT-lysine after hydrolysis.

Examinations of DNT-insulin made at pH 9·2 as described above (Exps. *A*/4, *A*/5 and *A*/9 of Table 1) showed that under these conditions very little, if any, substitution took place in the tyrosine residues. Reduction of the unhydrolysed protein derivative indicated only small amounts of reducible material in excess of the masked amino groups, but as the blank determinations were rather large, this indicates only that the number of *O*-DNT-tyrosine residues was less than 1 for each molecule of insulin. When Herriot's (1935) method was used to estimate the intact tyrosine of these derivatives, the estimates ranged between 9–10 mol. of tyrosine/mol. of insulin, compared with a value of 9 for the native protein.

Table 1. *The number of tyrosine residues masked in preparations of DNT-insulin made at* pH 9·2 *and* pH 10·4

Exp.	pH	Total number of amino groups masked/mol.	Tyrosine masked (mol./mol.) Reducible with $TiCl_3$	Tyrosine masked (mol./mol.) By Herriot's (1935) method
A/4	9·2	2·58	<1	—
A/5	9·2	3·19	<1	0
A/9	9·2	2·95	<1	0
D/2	10·4	1·51	7·4	—
D/3	10·4	1·92	2·6	2·4
D/4	10·4	1·77	4·7	6·8
D/5	10·4	1·93	6·0	6·1

The materials of experiments *D*/3, *D*/4 and *D*/5 (Table 1) were prepared in the following way. About 100 mg. of insulin were allowed to react with 2:4:5-TNT overnight at pH 10·4, the ethanol removed by extraction with ether and the pH adjusted to the isoelectric point. It was found that, after a certain amount of acid had been added and the precipitate (*D*/5) had been removed, more acid could be added without at first causing any further precipitation. However, after the pH had fallen to a lower value, more insulin derivative separated (*D*/4). When this had been centrifuged off, the addition of acid was continued and a third product isolated in a similar way (*D*/3). As can be seen from Table 1, the amounts of *O*-DNT-tyrosine present show a corresponding fall. That some tyrosine remained unmasked was demonstrated by hydrolysis in 6N-hydrochloric acid followed by paper chromatography.

Table 2 summarizes the approximate activities found for the treated insulin preparations and the distribution of the three DNT-amino-acids isolated from them. The activity was usually either very high, when it has been called 100 %, or very low, when it has been put as zero.

It is apparent that a change in pH has a marked effect on the distribution of DNT residues among the available amino groups. The fact that the terminal glycine amino group seems to react more readily than that of phenylalanine might be due to either steric hindrance within the insulin molecule, or to a chemical difference in reactivity between these residues when present in a peptide chain. In order to test the last possibility equimolar quantities of glycine, phenylalanine and 2:4:5-TNT were dissolved in 70 % ethanol saturated with pH 7·8 buffer. After reacting for 18 hr. the DNT-amino-acids were estimated as already described. It was found that phenylalanine had reacted more rapidly than glycine, the molar ratio of the DNT derivatives being 2·58. This experiment was repeated using the amino-acid esters. The phenylalanine derivative again reacted more rapidly, forming 2·64 mol. of DNT compound for every molecule of DNT-glycine ester.

It is noticeable that the amounts of DNT derivatives isolated from the treated insulins are in slightly different proportions even among experiments made under similar conditions (Table 2). The reason for this lack of agreement is not fully understood, but may be due to random variations of room temperature, at which these experiments were made.

The effect of the reaction medium on the activity of insulin was determined by making mixtures for each buffer used, the 2:4:5-TNT being omitted. Assays in rabbits showed that in no case had any detectable inactivation occurred.

DISCUSSION

These results show clearly that, in the first place, sufficiently extensive substitution with 2:4:5-TNT can destroy the activity of insulin, while in the second place, this substitution reaction follows a course dictated by the pH of the medium.

It will be seen from Table 2 that the activity of the partially arylated insulins was dependent on the number of amino groups masked, falling to zero when rather less than three residues had been

Table 2. *The distribution of DNT residues among the free amino groups of insulin, showing mols. of DNT amino-acid isolated/mol. of insulin*

(The pH quoted is an estimate of the value of the buffer in 70 % ethanol solution.)

Exp.	pH	Glycine (mol./mol.)	Phenylalanine (mol./mol.)	Lysine (mol./mol.)	Total of amino groups masked/mol.	Activity as approx. % of original
A/1	9·2	—	—	—	0·3	100
A/2	9·2	1·3	0·5	0·15	1·95	100
A/3	9·2	1·5	0·3	0·12	1·92	100
A/4	9·2	2·0	0·4	0·18	2·58	75
A/5	9·2	2·1	0·7	0·39	3·19	30
A/6	9·2	—	—	—	3·3	10
A/7	9·2	1·6	0·6	0·35	2·55	—
A/8	9·2	2·1	0·6	0·25	2·95	0
B/1	9·4	0·9	0·6	0·18	1·68	100
C/1	9·7	0·7	0·5	0·31	1·51	100
C/2	9·7	0·9	0·4	0·55	1·85	100
C/3	9·7	1·2	0·8	0·80	2·80	10
C/4	9·7	1·2	1·2	0·64	3·04	0
C/5	9·7	0·9	1·0	1·18	3·08	0
D/1	10·4	0·8	0·2	0·87	1·87	0
D/2	10·4	0·6	0·1	0·81	1·51	0
D/3	10·4	0·6	0·2	1·12	1·92	0
D/4	10·4	0·7	0·2	0·87	1·77	0
D/5	10·4	0·8	0·3	0·83	1·93	0

introduced. This seems to indicate that the effect of the masking residue is dependent on its size because, although the amino groups of insulin are unessential when tested with acetic anhydride (Fraenkel-Conrat & Fraenkel-Conrat, 1950), some at least are essential when the reagent is 2:4:5-TNT.

A noticeable feature of the results given in Table 2 is the suddenness of the fall in activity after just over two DNT residues have been introduced into each molecule of insulin. This is consistent with a steric effect which is insufficient to cause inactivation until more than two DNT groups are present. In no case was the amount of DNT-glycine isolated much less than 1 mol./mol. of insulin, and in experiments *A*/2, *A*/3 and *A*/4, it approached the maximum of 2 mol./mol. without marked loss of activity. The terminal glycine-amino groups are therefore relatively unessential, considered by themselves.

The separate effect of masking the phenylalanine and lysine residues cannot be decided from these experiments with any certainty because in preparations where much of either was labelled, considerable amounts of *O*-DNT-tyrosine were formed as well. Experiment *C*/2 shows that the lysine residues may not be intimately concerned with insulin activity, since in this case one group in every second molecule had been attacked without destruction of potency. In a similar way, from *C*/1 and *C*/2 it appears that the phenylalanine-amino groups cannot be individually connected with the hormone action, since if they were both involved the activity of these preparations would be only 50%. Even if only one of the phenylalanine residues is essential the activity of these derivatives should be no more than 75% of that of the original protein.

The experiments on the relative rates of reaction of 2:4:5-TNT with free glycine and phenylalanine indicate that the more rapid masking of the glycine terminal is unexpected. This is the more so since Schwartz & Lea (1952) have shown that when insulin combines with glucose, the phenylalanine couples more quickly than the glycine residues. However, it is significant that whereas the reaction with 2:4:5-TNT took place in 70% ethanol, Schwartz & Lea used a mixture freeze-dried from water at pH 7. The large increase in rate of reaction of lysine as the pH of the medium was raised may be due to the increased proportion of the uncharged form of the amino group in more alkaline solutions. A similar explanation would be applicable to the increase in speed of reaction of the tyrosine residues, as a higher pH would favour the dissociation of the hydroxyl group. According to Cohn & Edsall (1943) the pK value for the ionization of the phenolic group is 10·1 while that of the lysine ϵ-amino group is 10·5. The corresponding values for the free amino groups of glycine and phenylalanine are about 8·3 and 7·7 respectively. If this constant is a controlling factor, the phenylalanine should react slightly more rapidly than the glycine, as was found for the amino-acids and their esters. The inverse phenomenon during the reaction between 2:4:5-TNT and insulin suggests that the glycine terminal is sterically more readily available than the phenylalanine, but in view of the findings of Schwartz & Lea this question must remain open.

As no evidence has been found to support the view that the ϵ-amino groups of lysine must remain intact if hormonal activity is to be retained, the essential structure of the carboxyl end of the molecule remains unresolved.

SUMMARY

1. The application of 2:4:5-trinitrotoluene to the preparation of labelled derivatives of insulin has been examined.

2. The glycine amino terminals of insulin have been found to react more quickly than phenylalanine and these more rapidly than lysine.

3. The reaction with all the free amino groups and with the tyrosine phenolic groups was found to be markedly dependent on the pH of the medium.

4. By stopping the reaction after an appropriate time, insulin derivatives have been made which are soluble in water and which retain some or all of the activity of the original protein. The distribution of masked amino groups in such dinitrotolyl derivatives suggests that the free amino groups are not essential to the hormonal action of insulin.

Thanks are due to Mrs S. A. Simpson and her assistants for help with the test of insulin activity, while this work has been made possible only by the continued support of Prof. E. C. Dodds, F.R.S.

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Sedimentation and Diffusion Studies on Insulin: the Maximum Molecular Weight

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During a recent investigation of the sedimentation and diffusion constants of enzymic digests of insulin, comparative control experiments on the untreated, crystalline protein led to a lower value of the molecular weight than was expected. It was at first anticipated that the results would be explicable on the basis of the dissociation phenomena reported by Gutfreund (1948*a*, *b*) from osmotic studies, where the observed molecular weight was found to vary with the temperature, insulin concentration, and pH of the solution.

The osmotic method is well adapted for the study of dissociating systems, as it gives a (number average) value for the molecular weight directly, whereas the combination of sedimentation and diffusion measurements leads only indirectly to the molecular weight and may, in such systems, give a rather complex average. Nevertheless, in view of the present rather confused position concerning the molecular weights obtained from sedimentation and diffusion constants (previous values are summarized and criticized in the Discussion), it was felt that a limited study of the dissociation phenomena by these means, the accuracy and reproducibility of which have recently been established (Cecil & Ogston, 1948; Charlwood, 1952*a*; Creeth, 1952), would be rewarding, in that it should provide qualitative evidence of dissociation and, at least, should give a reliable value for the maximum molecular weight in the range in which the molecule is stable. This paper presents the results of such a study.

EXPERIMENTAL

Methods. Sedimentation constants were determined with the Spinco electrically driven ultracentrifuge, the details of experimental method and computation of results being exactly as described previously (Charlwood, 1952*a*, *b*). In one case, a determination was made at a low temperature (~7°); the rotor and cell were cooled to this temperature before being inserted in the rotor chamber, and the latter was kept cool during the run by periodic use of the refrigerator embodied in the machine. The temperature of the rotor fell by 1° during the run of approximately 2 hr. duration.

Diffusion measurements were carried out with the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948; Ogston, 1949*a*), previously described (Creeth, 1952), the only modification of the apparatus being the construction of an additional thermostating bath to surround the capillary exit tube from the cell and its ancillary boundary-forming device. By this means, all of the apparatus, with the exception of a short section of the capillary tube joining the two baths, is susceptible of accurate temperature control, and the need to work within about 1° of room temperature obviated. All determinations were made in duplicate, as has been the standard practice with this apparatus. In many cases it was desired to know if the diffusion coefficient varied with temperature in addition to the other variables studied; two pairs of runs were therefore performed on each solution, one at a temperature in the range 14–17° (referred to in the tables as 'lower temperature') and the other at about 28–30° ('higher temperature').

Although the operating temperature of the apparatus could be lowered to about 10°, by cooling the tap water passing through the copper coil in the main thermostating bath, this was not done as a routine as it involved the use of large quantities of ice. A temperature within the lower range referred to could be attained by the use of tap water without previous cooling.

All the observed diffusion coefficients have been corrected to the viscosity and absolute temperature of water at 20°, and are expressed in units of 10^{-7} cm.^{2} sec.$^{-1}$. Sedimentation constants were also corrected, and are stated in Svedbergs, i.e. 10^{-13} sec. units.

Materials. Work was largely performed on a particular sample of six-times recrystallized Zn-insulin obtained from Boots Pure Drug Co. Ltd.; other samples of crystalline material were obtained from Burroughs Wellcome and Co. Ltd., the British Drug Houses Ltd., and from Dr K. O. Pedersen of Uppsala. The latter sample was identical with the purified insulin used by Dr Pedersen in his work (1950), but had been stored at room temperature for 3 years. All solutions were dialysed in cellophan tubing, against about 100 vol. of buffer, for approximately 48 hr. before use; dialysis frequently had to be conducted at room temperature (about 20°) to prevent the insulin being precipitated. Where difficulty was encountered in dissolving insulin, the suspension was warmed to 35° for a few minutes. If precipitation subsequently occurred during dialysis, or the solution did not appear clear, the solution was centrifuged and the supernatant redialysed.

RESULTS

The sedimentation constant values obtained for the various samples of insulin investigated are shown, with the relevant experimental conditions, in Table 1.

It is evident from these results that there is no marked difference between the insulin samples of different origin. Between the values obtained at

Table 1. *Sedimentation constants for insulin*

(All solutions in phosphate buffer; $I = 0{\cdot}10$ except where stated.)

Material	Concentration (g./100 ml.)	pH	Temperature ($T°$)	$S_{20,w}$
Boots batch no. I 9633 B	0·4	7·4	17·0	3·10
Boots batch no. I 9633 B	0·4	7·4	25·9	3·11*
Boots batch no. I 9633 B	0·8	7·4	24·8	3·11
Boots batch no. I 9011 G	0·4	7·4	24·5	3·11
Boots batch no. I 11038 A	0·4	7·4	25·5	3·10
Burroughs Wellcome	0·4	7·4	22·0	3·15
B.D.H., batch no. 2083	0·4	7·4	21·8	3·13
Sample from Dr Pedersen	0·4	7·4	24·4	3·15
Boots batch no. I 9633 B	0·5	8·0	7·5	3·07
Boots batch no. I 9633 B	0·5	8·0	22·7	3·09
Boots batch no. I 9633 B	0·5	8·0	28·4	3·10
Boots batch no. I 11038 A	0·5	8·0	23·3	3·02

* Higher ionic strength; buffer composition: phosphate, $I = 0{\cdot}05$, sodium chloride, $I = 0{\cdot}25$.

pH 7·4 and those at pH 8, giving means of 3·12 and 3·07 respectively, there is a just significant difference ($0{\cdot}05 > P > 0{\cdot}02$). The run done at 0·8 % (w/v) concentration at pH 7·4 shows, in agreement with earlier observations (e.g. Gutfreund & Ogston, 1946; Pedersen, 1950), that there is no appreciable concentration dependence of the corrected sedimentation constant. Accordingly, the small difference between the two sets of figures can be ascribed to the effect of the pH change. It is also evident that, at pH 8·0, there is no temperature dependence of the sedimentation constant.

An attempt was made to simulate the conditions used by Oncley, Ellenbogen, Gitlin & Gurd (1952), who observed that the sedimentation constant of insulin increased as the ionic strength was raised from 0·10 to 0·68. The solubility of the insulin used here in phosphate-sodium chloride buffer, $I = 0{\cdot}60$, was too small, however, and the ionic strength had to be reduced to 0·30 before a suitable solution could be obtained. The sedimentation constant for this solution, however, was in good agreement with all others at this pH value.

In general, it has been found impossible to prepare solutions as concentrated as those used by Oncley *et al.* (1952); this may be due to the dissolution of the insulin directly, without prior solution in dilute acid, as has sometimes been used (Oncley, referred to by Waugh, 1948).

Following Jullander (1945) and Oncley *et al.* (1952), attempts were made in some cases to compute weight average sedimentation constants, S_w from the equation:

$$S_w = \frac{3S_m - S_t}{2}.$$

Here S_m is the sedimentation constant calculated from the movement of the median of the boundary, and S_t that from the mode. The latter corresponds to the most probable sedimentation constant, and is the one usually calculated. At pH 7·4, no appreciable difference between S_m and S_t could be found, and at pH 8 the case showing most boundary spread gave a value of S_w only 0·8 % lower than S_t, which is well within the experimental error. It was therefore concluded, for the purpose of subsequent calculations, that S_w and S_t were identical. This is in agreement with Jullander's general conclusions on the characteristics of sedimenting protein boundaries.

Table 2. *The effect of temperature variation on the diffusion coefficient of insulin at* pH 8·0

Insulin concentration 0·5 g./100 ml. $T°$	Phosphate buffer, $I = 0{\cdot}10$ $D_{20,w}$
10·7	8·81
14·5	8·67
20·0	8·81
27·8	8·80
28·3	8·81

Diffusion results. Except where stated otherwise, these refer exclusively to Boots insulin, batch I9633B. It was anticipated that the diffusion coefficients would be more sensitive to changes in particle size than the sedimentation constants (see, for example, Charlwood, 1952*b*). Accordingly, a wider range of variables was investigated, of which the first was the effect of temperature variation at a constant insulin concentration, ionic strength and pH; the results are shown in Table 2. These show immediately that there is no temperature dependence of the corrected diffusion coefficient under these conditions.

To determine whether temperature dependence occurred at other concentrations under these conditions (Gutfreund's (1948*a*) temperature-dependent molecular weight values referred to a much lower concentration as well as a lower pH), diffusion coefficients were determined over the concentration range 0·2–0·8 g./100 ml., at two temperatures (see

Experimental section). The results are shown in Table 3. The relation between the concentration and refraction increment was obtained by the use of the figure $1{\cdot}94 \times 10^{-4}$ for the specific refraction increment of insulin. This was calculated from the data given by Doty, Gellert & Rabinovitch (1952) using the equation given by Perlmann & Longsworth (1948). It is clear that over the whole range down to 0·3 g./100 ml. the diffusion coefficient is independent of concentration and temperature. The value at the lowest concentration is significantly higher, and doubtless reflects the dissociation which, from the osmotic measurements reviewed later, would be expected to occur in this region.

Table 3. *Variation of the diffusion coefficient of insulin with concentration, at* pH 8

(All solutions in phosphate buffer, $I = 0{\cdot}10$; concentrations expressed in terms of the refraction increment, v = number of wavelengths retardation; $v = 29{\cdot}36$ corresponds to 0·80 g./100 ml. solution.)

	$D_{20,w}$	
v	Lower temp.	Higher temp.
7·95	9·22	9·30
9·82	8·70	8·87
14·04	—	8·80
15·79	8·81	—
16·67	8·74	—
18·37	—	8·93
29·36	—	8·85

The values for the diffusion coefficient are much higher than those obtained by previous workers, and in view of Gutfreund's (1948*b*) evidence, that dissociation may occur outside the pH range 7·0–7·5, the diffusion coefficients of solutions of concentration approximately 0·4 g./100 ml. were determined over a limited range of pH; the results obtained are shown in Table 4.

Table 4. *Variation of the diffusion coefficient of insulin with* pH

(Insulin concentration = 0·4–0·5 g./100 ml. Phosphate buffers, $I = 0{\cdot}10$.)

	$D_{20,w}$	
pH	Lower temp.	Higher temp.
7·0	—	8·30
7·2	—	8·25
7·4	8·22*	8·29*
7·6	8·60	8·62
8·0	8·80	8·82

* Average values, see Table 5.

These results are in good qualitative agreement with the findings of Gutfreund (1948*b*), there being evidently no appreciable change of diffusion coefficient over the range pH 7·0–7·4, while above this a marked increase occurs, indicative of dissociation into sub-units. No significant difference is evident between the results obtained at the two temperatures.

At this stage, it appeared that the stability conditions for insulin had been established with sufficient precision for a more detailed study of the relationship between dissociation and concentration to be undertaken. The pH value of 7·4 was chosen, as at this pH the solubility is just sufficient for most of the solutions to be studied at the two temperatures.

The results obtained are shown in Table 5, from which it can be seen that, as at pH 8·0, the diffusion coefficient does not vary over the range 0·8–0·3 g./100 ml., but that below the latter concentration there is a rise indicative of the onset of dissociation. Again the influence of temperature, in the limited range in which study is possible, appears to be negligible.

Table 5. *Variation of the diffusion coefficient of insulin with concentration, at* pH 7·4

(All solutions in phosphate buffer, $I = 0{\cdot}10$. v = number of wavelengths retardation.)

	$D_{20,w}$	
v	Lower temp.	Higher temp.
5·93	9·45	—
6·65	9·59	9·55
7·20	8·97	9·10
8·76	8·74	8·74
9·45	8·86	8·96
9·80	—	8·77
11·01	8·40	8·60
12·07	8·23	—
13·98	8·21	8·25
17·65	—	8·34
25·18	8·23*	

* Run at 20·0°.

From these results it appears that the diffusion coefficient of insulin tends to the value of about 8·2 in the stability region. In order to determine whether this value really does represent the minimum attainable, diffusion experiments were performed under conditions which might be expected to favour further association. By lowering the ionic strength the solubility at low temperatures is increased, and the first of these auxiliary experiments was performed with an insulin concentration of 0·55 g./100 ml. and ionic strength 0·04 (pH 7·4). At 14°, the lowest attainable, this gave $D_{20,w} = 8{\cdot}51$.

The solution in buffer of $I = 0{\cdot}30$, referred to under the sedimentation experiments, gave $D_{20,w} \sim 8{\cdot}6$ (measured at 30·1°). This solution had to be dialysed at 35°, to avoid precipitation; to minimize bacterial action the period of dialysis was cut to 12 hr. It is possible, therefore, that this result is slightly high because of incomplete dialysis.

From these experiments, it appears that neither of these sets of conditions, namely, higher protein

concentration at a low temperature and low ionic strength, and high ionic strength and (perforce) high temperature, causes any reduction in the diffusion coefficient below the value obtained in the established stability range.

Table 6. *Diffusion coefficients for insulin of various origins*

(Insulin concentration 0·4 g./100 ml. phosphate buffer, $I = 0{\cdot}10$, pH 7·4, $T = 20$–$23°$.)

Description of insulin	$D_{20,\,w}$
Boots batch no. I 9633 B	8·25
Boots batch no. I 9011 G	8·20
Boots batch no. I 11038 A*	8·90
Burroughs Wellcome	8·82
B.D.H., batch no. 2083	8·69
Sample from Dr Pedersen	8·16

* This sample was more than 4 years old.

Finally, Table 6 shows the results obtained on insulin samples of different origin, together with the average value for Boots batch I 9633 B inserted for comparison. The conditions chosen for these experiments were such as to minimize dissociation, that is, an insulin concentration of about 0·4 g./100 ml. at pH 7·4. The results show clearly that considerable differences in diffusion coefficient may occur in samples of different origin, but that there can be little doubt that two of the three Boots samples and the purified sample from Dr Pedersen, are directly comparable. The rather wide variations in diffusion coefficient are in contrast to the sensibly constant values of the corresponding sedimentation constants, and form another example of the relative insensitivity of the latter characteristic.

All the above diffusion coefficients have been calculated from the movement of the outermost minimum in the record of the interference pattern, the position of which is very largely determined by the magnitude of the refractive index gradient in the centre of the boundary (Ogston, 1949*a*). The values obtained accordingly correspond to the D_A values obtained by the Lamm (1937) scale, or diagonal schlieren, methods, and, as stated by Ogston (1949*b*) for a mixture of components each diffusing ideally, the mean diffusion coefficient, D, obtained by this procedure, is that average given by Quensel (1942):

$$\frac{1}{\sqrt{D}} = \Sigma \frac{\alpha_i}{\sqrt{D_i}},$$

where α_i is the proportion, in terms of refractive increment, of component of diffusion coefficient D_i.

It has long been recognized that the ratio of D_m (the diffusion coefficient obtained from the second moment of the gradient curve) to D_A is some measure of the homogeneity of the diffusing substance (Gralén, 1944), and where the specific refractive increments of all components are equal (as is almost certainly the case with the insulin monomer-polymer system), D_m is the weight average diffusion coefficient of the mixture:

$$D_m = \Sigma \alpha_i D_i.$$

A method of determining D_m from the Gouy interference pattern has recently been developed by Ogston (1949*b*). It is doubtful whether it may be applied reliably to solutions of less than about 20 wavelengths retardation, but for stronger solutions, it is only slightly less accurate than the procedure used to give D_A. Accordingly, D_m values were computed for the most concentrated insulin solutions referred to in Tables 3 and 5. The values obtained were 8·90 (compared with $D_A = 8{\cdot}78$) at pH 8·0 and 8·16 ($D_A = 8{\cdot}23$) at pH 7·4.

The difference between the two values at pH 7·4 is negligible. At the higher pH, the difference is only about 2·5 % and is still well within the experimental error. Accordingly, these figures cannot be used to give a reliable value of the degree of dissociation under these conditions, and an attempt to calculate the maximum possible degree of dissociation, by ascribing a probable value to the error attendant upon D_m and assuming the value of 8·2 for the diffusion coefficient of the polymer, was abandoned, as it led to a value of < 10 for the diffusion coefficient of the monomer. Such a low value must be considered unlikely, both on general grounds and in view of the results in acid solutions obtained by Fredericq & Neurath (1950).

DISCUSSION

Earlier values of the molecular weight of insulin show considerable discrepancies. In historical order, they may be summarized as follows: Sjögren & Svedberg (1931) using the equilibrium ultracentrifuge obtained S_{20} (uncorrected) = 3·47, and $M = 35000$; Polson (1939) determined the diffusion coefficient finding $D_{20,\,w} = 8{\cdot}2$: from this figure and Sjögren & Svedberg's value for S, an independent value of $M = 40900$ was calculated. The next evidence was crystallographic: Crowfoot (1938) showed that the weight of the dry unit cell was 36000 (this figure has been corrected from the original, using modern values for the proportion of water in 'dry' insulin, following Gutfreund (1948*a*)) and that the cell had trigonal symmetry, thus indicating that the molecular weight in the solid state was 36000, and that this referred to an aggregate of 3, or $3n$, identical units. Miller & Andersson (1942), using the sedimentation velocity method, found $S_{20,\,w} = 3{\cdot}55$ for the pH range 7·0–7·5. Diffusion measurements in two different forms of apparatus gave a mean value $D_{20,\,w} = 7{\cdot}53$. Using the value of 0·749 for the partial specific volume

(Sjögren & Svedberg, 1931), these workers calculated $M = 46\,000$. Gutfreund & Ogston (1946), however, obtained a lower sedimentation constant than Miller & Andersson (1942), finding $S_{20,w} = 3{\cdot}34$, a value which was constant over the pH range 6·7–9·0.

Gutfreund's osmotic pressure measurements (1948*a*, *b*) showed that, at pH 7·0–7·5, the molecule was stable in the concentration range 0·4–1·0 g./100 ml., but that dissociation, leading to a lower average molecular weight, occurred at concentrations lower than about 0·3 g./100 ml. Dissociation, moreover, was a function of the temperature in the concentration region 0·10–0·16 g./100 ml. Measurements in acid and alkaline solutions showed that the range of stability was rather narrow, dissociation occurring at pH values above 7·5 and below 4. In the first of these papers, Gutfreund gave a value for the diffusion coefficient of insulin of 7·0, which combined with Gutfreund & Ogston's (1946) value for the sedimentation constant, gave $M = 47\,000$. Gutfreund's final conclusion was that the molecular weight of the sub-unit was 12 000, that three such units formed the molecule in the dry state, but that four units formed a stable aggregate, of molecular weight 48 000, in neutral solution.

This conclusion was criticized by Pedersen (1950), who presented further evidence from dynamic measurements: values of $S_{20,w} = 3{\cdot}46$ and $D_{20,w} = 7{\cdot}59$, with a new value for the partial specific volume $\bar{v} = 0{\cdot}735$ were used to calculate $M = 42\,000$. Pedersen also pointed out that Gutfreund & Ogston's (1946) $S_{20,w}$ and Gutfreund's (1948*a*), $D_{20,w}$ values, if used with the new value for $\bar{v}$, gave $M = 44\,000$.

Fredericq & Neurath (1950) found $S_{20,w} = 3{\cdot}45$ at pH 7·0 and 0·25 g./100 ml. concentration; other values, referring to acid solutions, were taken to indicate that the molecular weight of the sub-unit was 6000, a view which was subsequently modified (Tietze & Neurath, 1952) to agree with Gutfreund's (1952) conclusion that the relevant figure was 12 000.

Oncley *et al.* (1952) came to the same conclusion regarding the size of the sub-unit, but concluded on the basis of sedimentation velocity measurements that probably only three units associated in neutral solution, pointing out that the value of 48 000, resting largely on the low figure used for the diffusion coefficient, leads to a frictional ratio higher than would be expected from viscosity measurements. At pH 7·3, the value of $S_{20,w}$ reported by these workers was about 3·4, but in solutions of very high ionic strength (0·68), this was increased to over 4; the latter figure, together with a value referring to acid solutions, was used to describe the equilibrium between monomer and polymer in acid solutions.

As Gutfreund (1948*a*) stated, some of the discrepancies in the dynamic quantities noted above may represent real variations in molecular weight, originating in dissociation of the substance with concentration, temperature, or pH. However, as most of the investigations recorded were performed in the known stability range, other grounds of criticism should be considered. Recent work (Cecil & Ogston, 1948, 1949; Kegeles & Gutter, 1951; Charlwood, 1952*a*; Creeth, 1952) has shown that the sedimentation constants of several proteins published by the Swedish workers are somewhat high, and this has been confirmed for insulin by Pedersen (1952). Moreover, the work of Gutfreund & Ogston (1946) was performed before the introduction of the standard conditions for running the oil turbine, and the results are hence likely to be some 2 or 3 % too high (Ogston, 1952). Similar criticism can be applied to the American workers' values for insulin.

For the diffusion coefficients, Miller & Andersson's (1942) values show a total variation of nearly 20 %, whereas for the much lower value quoted by Gutfreund (1948*a*), no details of method, concentration or other experimental conditions were given. Pedersen's (1950) values show D_m about 4 % lower than D_A, but on theoretical grounds any inequality must be in the opposite sense.

Molecular weights determined from these dynamic data must accordingly be subject to comparatively large errors, but, on the whole, tend to give a lower value than indicated from the osmotic data.

The mean value obtained in this investigation for the sedimentation constant of insulin within its stability range, 3·12, is considerably lower than the figures cited above, the nearest being that obtained by Gutfreund & Ogston (1946), after correction by the factor 0·978 given by Cecil & Ogston (1948), which is 3·27, and the revised value of 3·30 given by Pedersen (1952). As the Spinco ultracentrifuge used here has been shown to give the same results as the oil turbine at Oxford (Charlwood, 1952*a*) when the latter is run under standard conditions, there is no reason for doubting the validity of the value 3·12. As Oncley *et al.* (1952) pointed out, in the sedimenting boundary of any insulin solution there must be some levels at which the concentration is low enough to permit dissociation. In view of the boundary characteristics described in the experimental section, it is unlikely that any error from this cause is associated with the above value.

From the diffusion data, it appears that the diffusion behaviour in the stability range approximates to the ideal ($D_A \sim D_m$) and that the constant has a value of about 8·2. This agrees with Polson's (1939) determinations, but differs quite widely from the other results. A possible reason for the discrepancies has been advanced by Pedersen (1952); if the solutions have been prepared by slight warming, it is very easy to obtain supersaturation, and such solutions, as Gutfreund (1948*a*) noticed,

tend to precipitate on standing. If, during the rather protracted duration of a diffusion experiment by the classical procedures, material tends to come slowly out of solution but remains in suspension, low results may be anticipated. Such behaviour would not be expected to influence the sedimentation similarly, as any suspended material would be very rapidly removed. During the comparatively short duration of the diffusion experiments described here, it is unlikely that phenomena attendant upon supersaturation would have an appreciable effect, especially in view of the precautions outlined in the experimental section.

If these values for the sedimentation and diffusion constants are inserted into the Svedberg equation

$$M = \frac{RTS}{D(1-\bar{v}\rho)}$$

(where $\bar{v}$ is the partial specific volume of the protein and ρ the density of the medium to which the sedimentation and diffusion constants are referred, namely water at 20°) one obtains the molecular weight values of 34800, if $\bar{v}=0{\cdot}735$ (Pedersen, 1950), or 36700 on the older figure, $\bar{v}=0{\cdot}749$ (Svedberg & Pedersen, 1940). Even lower values for $\bar{v}$ have recently been claimed (Oncley *et al.* 1952), the use of which would, of course, give a lower figure for the molecular weight, but in the absence of detailed evidence the use of the more conventional value is probably to be preferred. The result of this investigation, therefore, is to give the maximum molecular weight of insulin as approximately 36000. The frictional ratio is 1·20. The values for the dynamic quantities used here do not, of course, refer to zero concentration, as is theoretically required. However, in view of the virtual independence of concentration shown by the sedimentation constant, and the apparently similar behaviour in diffusion demonstrated by the results (down to 0·3 g./100 ml.) of Table 5, it is unlikely that if the extrapolation were possible it would yield a greatly different value of the molecular weight.

The value of 36000 is not in agreement with the recent work of Doty *et al.* (1952), in which evidence for the existence of the tetrameric form of insulin was adduced from light-scattering studies at so low a pH as 3·2. The work of Gutfreund & Ogston (1946) at this pH indicated that the sedimentation constant was slightly lower than at pH 7, and Gutfreund's osmotic data (1948*b*) confirmed the occurrence of dissociation in this region, at the relevant concentration. There is clearly scope for the extension of diffusion work to this pH region.

The reason for the discrepancy between the values obtained by the osmotic and dynamic methods is not clear. The number average molecular weight given by the former method should, in the case of a mixture, be a lower value than that given by the latter, for, as Jullander (1945) has shown, the combination of weight average sedimentation and diffusion constants, while not giving a weight average molecular weight, will generally give a value between the weight and number averages.

Apart from this, the evidence concerning the dissociation of insulin with concentration and pH is in good qualitative agreement with the results obtained by Gutfreund. While the limitations of conclusions formed from the rather restricted range of the results must be borne in mind, it does appear that temperature has little effect on the degree of dissociation under the conditions of the experiments reported here. This behaviour bears a superficial similarity to that discussed by Klotz & Curme (1948) (see also Fiess & Klotz, 1952) for the binding of copper ions to bovine serum albumin, where the assumption of a non-temperature-dependent electrostatic energy of interaction led to an expression for the binding in agreement with experimental observations. Oncley *et al.* (1952), however, found for insulin that the sedimentation behaviour could be explained on the basis of a repulsive electrostatic force acting together with a nearly constant attractive force of unspecified origin. Gutfreund (1952) also concluded that at low pH values, where the sub-units are strongly positively charged, dissociation would be favoured unless the ionic strength was high. In view of the inability to duplicate the higher sedimentation constants reported by Oncley *et al.* (1952) for solutions of higher ionic strength, it is probably unwise to speculate on the nature of the interaction in neutral solutions, beyond the observation that the lack of temperature-dependence indicates that the free energy of the aggregation-disaggregation reaction is associated largely with the entropy change.

SUMMARY

1. The sedimentation and diffusion characteristics of solutions of crystalline insulin have been investigated in the pH range 7–8; the variation of diffusion coefficient with concentration and pH confirms the evidence of dissociation advanced from other studies.

2. Temperature change, within the range 14–30°, has no apparent effect on the dissociation equilibrium.

3. The substance is apparently stable in the range pH 7·0–7·4 and at concentrations greater than 0·3 g./100 ml. Under these conditions, the sedimentation constant is 3·12 and the diffusion coefficient about 8·2. These values differ from previously accepted values.

4. The diffusion and sedimentation results have been combined to give a maximum molecular weight of about 36 000 for this protein, in agreement with X-ray data on the solid substance.

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The Metabolism of Collagen from Liver, Bone, Skin and Tendon in the Normal Rat

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In a previous communication (Neuberger, Perrone & Slack, 1951) the metabolism of collagen from normal rat tendon was studied. These experiments have now been extended to include collagen obtained from tendon, skin, bone and liver. Some of the results have been the subject of a preliminary communication (Perrone & Slack, 1951).

EXPERIMENTAL

Treatment of animals and administration of labelled glycine

Male albino rats of the Institute stock were used throughout. The animals consisted of three groups of rats: (1) old rats with body weight ranging from 350 to 390 g., all being more than 14 months old, in two series of four rats and three rats respectively; (2) young adults with body weight ranging from 200 to 250 g. in two series of four and five rats, and a further series of young adults of known age in two groups of three rats each; (3) young rats with body weight ranging from 50 to 70 g. in two series of five and three rats respectively.

All animals were apparently in normal health and had not been used for other experiments. Throughout these experiments they were given the Institute stock diet. Each rat received 10 μc./100 g. initial body weight of glycine labelled with ^{14}C in the methylene position. The specific activity of the glycine was 1 mc./m-mole. The required dose was weighed on a microbalance, dissolved in 1 ml. of distilled water and administered by intraperitoneal injection. The animals were killed by stunning at varying times after the injection.

Growth of animals. The old rats showed no marked or consistent changes in body weight (Table 1). The young

adults showed some increases in weight during the experiments, the rate varying somewhat with different animals (Tables 3 and 4). The young rats all increased their body weights very markedly during the experiments (Table 2).

Preparation of samples

Liver and muscle proteins. These were prepared as described previously (Neuberger *et al.* 1951).

Tendon collagen. This was prepared as described (Neuberger *et al.* 1951).

Skin collagen. After shaving the rat to remove hair, the skin was freed from subcutaneous tissue and minced in a meat mincer. It was then immersed for 48 hr. in 5 times its own volume of 20% (w/v) urea. The residue was repeatedly washed in changes of distilled water. The collagen in the residue was converted to gelatin by boiling under reflux for 24 hr. with distilled water. After filtration through Whatman no. 42 paper the solution was reduced in volume by evaporation *in vacuo* on a water bath; acetone was added until no further precipitation occurred and the mixture was then left overnight at 0°. The gelatin was centrifuged off and dried in a desiccator. Samples were hydrolysed by boiling under reflux in 5·8N-HCl for 24 hr.

Liver collagen. The fresh liver was minced with scissors and then ground with sand in a mortar. After extraction for 48 hr. at 4° with 20% (w/v) urea, the residue was repeatedly washed with distilled water; the gelatin hydrolysate was obtained by the same means as that from skin.

Bone collagen. After removal of the head the rat carcass was roughly dissected free from muscle and connective-tissue masses. The carcass was broken up, boiled briefly in water and then rinsed in cold water. It was soaked for 24 hr. in 20% (w/v) urea at 4°. After repeated washing in distilled water the bones were pounded in a mortar and defatted by extraction with 50% (v/v) ether/ethanol. The bony residue was then treated with trypsin (British Drug Houses Ltd.) for 48 hr. at 37°. After repeated washing in distilled water the collagen matrix was converted to gelatin and hydrolysed as described above.

Isolation of 2:4-dinitrophenyl-(DNP-) glycine and radioactivity measurements

The hydrolysates were converted to the 2:4-dinitrophenyl derivatives and the DNP-glycine isolated as described (Neuberger *et al.* 1951). The radioactivities of the samples were also measured as previously described. All results were expressed as counts/min./2 sq.cm. disk of 'infinite thickness'.

RESULTS

Changes in the specific activities of glycine obtained from the mixed proteins of liver and muscle

The specific activities found in glycine obtained from the liver- and muscle-protein samples are very similar to those found previously (see Neuberger *et al.* 1951). Some additional information on the effects of growth and age has been obtained and this will be discussed later.

Changes of specific activity of the glycine obtained from the collagen of liver, bone, skin and tendons

The specific activities of the glycine isolated from hydrolysates of collagens obtained from the four tissues of the old rats at intervals of time ranging from 1 to 7 weeks after administration of the labelled glycine are shown in Table 1. In this series of experiments glycine was not isolated from the mixed proteins of liver or plasma, but in other experiments in which similar amounts (in terms of radioactivity) of [α-^{14}C]glycine were administered into rats of the same strain and age, the DNP-glycine samples obtained from the proteins of the internal organs 1 week after injection had activities of 1100–1700 counts/min./2 sq.cm. By comparison the activities of the DNP-glycine samples obtained from the various collagens were extremely low, 109 counts/min./2 sq.cm. being the highest. The variation in the radioactivities was considerable, but bone collagen appeared to be more active than collagen from skin, tendon or liver. The initial activity appeared to change little, at least up to 7 weeks.

In the very young rats (ages at time of injection 4–6 weeks) the initial activities of the glycine samples from all four types of collagen specimens were very high (Table 2) and not very much lower than corresponding values for muscle or even mixed liver proteins (see Neuberger *et al.* 1951, Table 4). It appears that in young rats differences between tissues with respect to rate of incorporation of labelled amino-acids have largely disappeared within 1 week and what is in fact mainly observed,

Table 1. *Specific radioactivities of 2:4-dinitrophenylglycine samples obtained from collagen isolated from liver, bone, skin and tendon of old rats injected with* 10 μc./100 *g. body weight of* $NH_2{}^{14}CH_2.COOH$

Rat no.	Body weight Initial (g.)	Body weight Final (g.)	Duration of experiment (weeks)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of collagen of: Liver	Bone	Skin	Tendon
121	362	370	1	38	80	34	—
122	386	378	2	29	—	15	—
123	394	390	3	34	62	40	20
105	358	371	3	42	101	14	24
106	390	370	3	—	79	22	12
101	373	355	7	—	109	28	15
102	385	380	7	—	88	—	18

Table 2. *Specific radioactivities of 2:4-dinitrophenylglycine samples obtained from collagen isolated from liver, bone, skin and tendon of young rats injected with 10 μc./100 g. body weight of* $NH_2{}^{14}CH_2.COOH$

(The figures in brackets are corrected specific radioactivities. These corrected figures were obtained by multiplying the observed radioactivity by ratio of final body weight to initial body weight.)

Rat no.	Body weight Initial (g.)	Body weight Final (g.)	Duration of experiment (weeks)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of collagen from: Liver	Bone	Skin	Tendon
115	79	115	1	681* (908)	670* (893)	—	621 (905)
116	80	97	1			—	663 (804)
139	65	94	1	686 (992)	622 (900)	591 (855)	—
150	69	96	1	645 (898)	665 (925)	562 (782)	—
62	62	102	2	418* (726)	480 (789)	361 (594)	324 (533)
140	60	110	2		451 (827)	340 (623)	—
141	65	127	3	385 (752)	401 (784)	321 (627)	—
100	50	160	8·5	235 (752)	220 (704)	—	193 (617)

* Pooled samples.

Table 3. *Specific radioactivities of 2:4-dinitrophenylglycine samples of collagen obtained from liver, bone, skin and tendon of young adult rats injected with 10 μc./100 g. initial body weight of* $NH_2{}^{14}CH_2.COOH$

Rat no.	Body weight Initial (g.)	Body weight Final (g.)	Duration of experiment (days)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of collagen of: Liver	Bone	Skin	Tendon
124	239	239	0·5	474	—	22	—
1	237	237	1	560*	—	41	46
125	141	143	1		—	—	—
126	209	212	3	345	362	34	—
2	233	242	7	290	351	28	68*
127	216	221	7	—	—	—	
3	203	229	14	271	312	25	58*
128	240	251	14	—	—	—	
4	206	252	21	252	294	—	159*
130	212	249	21	—	—	—	
5	205	236	35	260	290	—	174

* Pooled samples.

at this time and later, is growth or net increase of protein and not the turnover of body substance already laid down.

With young rats the specific activities of the collagen-glycine samples decreased markedly with lengthening of the interval between injection of glycine and killing of the animal. This decrease can be due to the dynamic state of collagen in young animals and/or to the deposition of collagen of decreasing isotope content in the later stages of the experiment ('growth dilution'). This point has been fully discussed already (Neuberger *et al.* 1951); the rough corrections which have been made assume that practically all the isotope is introduced into the collagen at the beginning of the experiment and that the total amounts of the various collagens in the rat are linearly proportional to body weight. The first assumption would certainly be incorrect for adults, but may be justified as a very crude approximation in very young rats in which the initial isotope content of the collagen glycine is relatively high. We have not found any data on changes of collagen content with age for such tissues of the rat as have been investigated in the present work, but the results of Lowry, Hastings, Hull & Brown (1942) indicate that there is no increase in the collagen content of muscle until the rat has passed reproductive age. The observed specific activities were therefore multiplied by the ratio of final to initial body weight. All the 'corrected' figures for young rats (Table 2) for the activities of glycine from collagen of bone, liver, skin and tendon show some fall during the first 3 weeks. Data for later periods in these young rats are scanty, but the few figures available suggest that a further decrease in activity in collagen after 3 weeks is slow. As noted previously, the activities of glycine from liver and mixed muscle proteins, especially the former, fall markedly with time in rats of all ages.

The activities found in the young adult rats are more difficult to interpret. Table 3 shows the activities of glycine from the collagen of the four tissues in young adult rats of weight range 200–250 g., but

Table 4. *Specific radioactivities of 2:4-dinitrophenylglycine samples of collagen obtained from liver, bone, skin and tendon of young adult rats of known age injected with 10 μc./100 g. body weight of* $NH_2{}^{14}CH_2.COOH$ *and killed 1 week later*

Rat no.	Age at death (days)	Body weight Initial (g.)	Body weight Final (g.)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of collagen of Liver	Bone	Skin	Tendon
5V†	103	273	274	185	208	40*	59
6G	103	264	274	174	221	72	40
7V	103	270	285	231	113	94	25
6R	151	317	318	174	127	81	11
7G	151	321	325	162	170	94	17
8R	151	318	327	155	176	86	34

* Derived from skin from the tail of the rat.
† Letters V, G, R indicate litter mates.

whose exact ages were not known. It will be seen that bone and liver collagen were fairly active and were actually 4–10 times as active as the collagen of skin and tendon. The few figures available for liver collagen suggest some active turnover during the first week. After the first week there appeared to be no appreciable loss of activity in any of the collagen fractions. A further group of young adult rats of known age was studied, glycine from the collagen of liver, bone, skin and tendon being obtained 1 week after injection of the radioactive glycine (Table 4). In addition, liver- and muscle-protein glycine were isolated, the results being similar to those reported previously. It will be seen (Table 4) that the increases in body weight during the week of the experiment are not large in either of the two series of three rats, though the rats aged 151 days were considerably heavier than those aged 103 days. Although there was considerable variation in individual figures, the glycine from bone and liver collagen was found to be considerably more active than that from skin, whilst tendon collagen was least active. Furthermore, the activities of all the collagen samples, with the exception of that from skin, were on the average lower in the rats aged 151 days than those in rats aged 103 days. A similar apparent decrease in activity with increasing age was seen in the glycine from liver and muscle protein.

DISCUSSION

Some general considerations on the interpretation of isotope data relating to protein metabolism

In this work and in an earlier paper (Neuberger *et al.* 1951) it was found that the injection of an amount of [α-^{14}C]glycine which produces a high isotope content in the mixed proteins of liver and plasma leads to a relatively slight labelling in the glycine residues of various collagens of the adult rat. The deduction made, that collagens as a group are metabolically almost inert or have turnover rates which are small by comparison with those of liver and even those of the mixed proteins of muscle, is, however, based on certain assumptions which have also been implied by other workers in the field of protein metabolism, but which have not been clearly stated. Isotope methods, as used at present, can only detect such changes in protein molecules as consist of a hydrolysis to amino-acids and complete synthesis of the protein from free amino-acids. It is possible that a protein may be broken down to peptides and these may be preferentially utilized for the synthesis of the original protein or for the synthesis of a different protein. If the hydrolysis or synthesis of such peptides from amino-acids is slow relative to the rate of synthesis of the protein, little incorporation of the labelled amino-acid will be observed. The protein may also be changed in other ways not involving the breaking of peptide linkages, or one protein may be transformed into another without the production of free amino-acids or the severance of covalent bonds. In all these cases degradation or synthesis of protein may not be reflected in changes of isotope content. It is concluded, therefore, that any statement on protein metabolism made on the basis of isotope results can refer only to such changes in which amino-acids are produced or in which amino-acids are the starting material of protein synthesis.

A further assumption which is generally made is that the amino-acids present in cells and arising from the reversible breakdown of proteins equilibrate readily with the labelled amino-acid administered. This is unlikely to be completely true. When a labelled amino-acid is fed or injected the isotope content of the labelled compound will be very high, immediately after administration, in the blood and in the intracellular spaces of some tissues, but will decrease rapidly in a few hours owing to mixing with a larger 'pool' and to various metabolic reactions (Arnstein & Neuberger, 1951). The maximum isotope content observed in a particular protein will thus not only be a function of the dose of isotope and the turnover rate of the protein in

question, but will also depend on the rate at which the labelled amino-acid penetrates to the site where the protein under investigation is synthesized. Permeability barriers may prevent complete equilibration between the intracellular amino-acid and the amino-acid present in the blood or extracellular space and thus the amino-acids liberated *in situ* by protein breakdown may be preferentially used for synthesis. Such permeability barriers may account, at least to some extent, for the relatively low maximum isotope contents found in brain and muscle, but it is unlikely that they will explain the extremely low values found with some collagens. For permeability factors to be rate-determining it is necessary that the rate of penetration of an amino-acid to the site of synthesis should be smaller than the rate of incorporation of the amino-acid into the protein. With collagens, the rate of incorporation of labelled glycine is so low that it is almost certain that, at least in the rat, it is the rate of protein synthesis as defined above which determines the overall rate of isotope incorporation.

Purity of the collagen samples

Another, less general, point which has to be considered is the purity of the collagen specimens. Collagens may be defined as a group of proteins, present mainly in fibrous form in the extracellular spaces of most or all tissues, showing a typical pattern under the electron microscope and having, at least in the case of mammalian collagens, an unusual amino-acid composition characterized by high glycine, proline and hydroxyproline content, presence of hydroxylysine, absence of tryptophan, and low content of other aromatic amino-acids, cystine and methionine. Collagen samples prepared by the methods used in the present work gave negative tests for cystine and tryptophan, had a very weak and indistinct absorption in the ultraviolet (265–295 mμ.) and contained between 13·5 and 14·0% hydroxyproline as measured by the method of Neuman & Logan (1950). Paper chromatograms of hydrolysates of these samples revealed the presence of only minute traces of phenylalanine and tyrosine. It would appear, therefore, that these samples were reasonably pure, but it is impossible to assert that the method of preparation has completely eliminated all proteins other than collagens. With skin, for instance, it was found (Harkness & Neuberger, 1952) that the glycine isolated from the soluble proteins had, soon after administration of the labelled compound, a high isotope content similar to that of glycine from plasma proteins, and the presence of even a small amount of such proteins in our early collagen specimens would give misleading results. With samples obtained more than 1 week after administration contamination would be somewhat less serious since the labelling in the soluble proteins decreases rapidly with time. Moreover, the glycine content of collagen is about 5–10 times greater than that of most globular proteins and it is therefore unlikely that the glycine derivative isolated contained more than 1% of material derived from sources other than collagen. It is probable that contamination was much less than this figure. It is suggested, therefore, that the radioactivities measured represent substantially the activities of the glycine residue in collagens. It may be mentioned, however, that 'procollagen' is probably included since it is not soluble in urea.

Comparison of the extent of isotope incorporation into different collagens of the adult rat

In the earlier paper (Neuberger *et al.* 1951), it was reported that the glycine isolated from the collagen of the tendon of the adult rat was almost completely inactive, and this finding is confirmed by the present work (Tables 1 and 4). The conclusion which has been put forward tentatively, that this may apply to collagens generally, has, however, to be slightly modified. Although the tendon samples were obtained from the limbs, feet and backs of the animals, as well as the tails, dissection is not easy and at least half of the sample consisted of tail tendon. Since the tail has a lower temperature, and therefore presumably a lower rate of metabolism, the observed activities of the mixed tendon-collagen samples may be particularly small. In this connexion it is of interest that one sample of skin collagen which was taken from the tail had a somewhat lower activity than samples taken from other parts of the body (Table 4). Collagens from skin and liver had definitely higher activities in adults than tendon collagen and must be presumed to turn over somewhat faster. The collagen of bone had the highest activity both in the very old rats (Table 1) and in the animals aged 103 and 151 days (Table 4), whilst in the group of young adults, the age of which was not known, bone and liver collagen had similar, moderately high, activities as compared with the very low activities of skin and tendon collagen (Table 3). The consistently high relative activity of bone collagen in all age groups suggested that the matrix of bone is replaced continuously at a slow rate, and this is in accordance with the observation that the epiphyses of the rat remain open during the life of the animal.

The collagen in certain organs of the rat increases in old age (Lowry *et al.* 1942; Myers & Long, 1946) and the possibility was considered (Neuberger *et al.* 1951) that such incorporation of isotope as was observed in the very old rats was due to a slight net increase of collagen and not to reversible degradation of material already laid down. The fact, however, that similar values for the various collagens

were found in the old animals and in rats aged 103 and 151 days suggests that collagen is not completely inert. The differences now observed between different collagens also indicate probably that the collagen molecules are continuously but very slowly replaced, even in the adult animal. But compared with liver, kidney or even muscle, turnover rates are very low, with the possible exception of bone collagen, and the contribution which is made by this group of extracellular proteins to the 'dynamic state' of the body is small.

The metabolic activity of collagens in young animals

In rapidly growing rats the labelled glycine is used for collagen synthesis, as shown by the high activities of the DNP-glycine obtained soon after administration of the labelled compound. The sharp decrease in specific activity observed with later samples (Table 2) is probably mainly due to the fact that the activity of the free glycine in the body decreases sharply within hours of administration. Since growth continues, collagen of rapidly decreasing activity is deposited thus leading to a loss of specific activity. The 'corrected' results (Table 2) indicate that there is a decrease not only in specific activity, but also in total radioactivities of all four collagen fractions. If collagen were inert, total activity should have increased. It seems likely, therefore, that collagen, including that of tendon and skin, is metabolically more active in young rats than in adults. This may be associated with the relatively large amounts of 'procollagen' found in growing animals (Bresler, Finogenow & Frenkel, 1950).

SUMMARY

1. [α-^{14}C]Glycine was injected into rats of varying ages. The animals were killed at different intervals of time and samples of mixed proteins of liver and muscle and of collagen from skin, tendon, bone and liver were obtained. Dinitrophenylglycine was isolated from the hydrolysate of these proteins and its radioactivity determined.

2. In rats more than 14 months old, the activities of the glycine samples obtained from all the collagen fractions were very low, bone collagen showing the highest values. In young adults which were still growing slowly, low activity was observed with skin and tendon collagen, but appreciably higher values were obtained with collagen from bone and liver. With young, rapidly growing rats initial activities were high with all collagen fractions.

3. The difficulties inherent in the interpretation of isotope data in relation to protein metabolism are discussed, and it is concluded that only such changes can be detected as involve participation of free amino-acids in protein turnover. The criteria of purity of collagen are also examined.

4. Subject to the limitations mentioned the results suggest that, even in the adult rat, collagen fibres are continually, but very slowly, replaced, the turnover rates being highest for collagen from bone. It is likely that in young rats this change is somewhat faster. However, in all age groups all collagens showed turnover rates which are low compared with those of intracellular proteins.

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Differentiation of Ribonucleic Acids by Inhibition of Streptococcal Deoxyribonuclease

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It has been shown (Bernheimer & Ruffier, 1951) that physical disruption of streptococci belonging to Lancefield groups A, B, C and D results in the liberation of a substance which partially inhibits the depolymerizing action of group A streptococcal deoxyribonuclease. One of the striking characteristics of the inhibitory substance is its specificity, for although it inhibits the deoxyribonuclease produced by group A streptococci, it fails to inhibit deoxyribonucleases prepared from pancreas, barley, yeast or even those of streptococci belonging to groups B and C. The inhibitor has been identified as streptococcal ribonucleic acid. There arises the question of whether the inhibition of group A streptococcal deoxyribonuclease is an effect associated specifically with ribonucleic acid of streptococcal origin or whether ribonucleic acids, regardless of source, will inhibit the enzyme. The only information bearing on this point is the result of an experiment in which yeast nucleic acid was found not to inhibit (Bernheimer & Ruffier, 1951). The present study is concerned with the capacity of ribonucleic acids, prepared from diverse sources, to inhibit the streptococcal enzyme.

METHODS

The methods of estimating deoxyribonuclease activity and inhibition were identical with those used by Bernheimer & Ruffier (1951). Inhibition of depolymerizing action of deoxyribonuclease was estimated by measuring the rate of decrease in viscosity of sodium deoxyribonucleate solution by streptococcal deoxyribonuclease (*a*) in the presence, and (*b*) in the absence of solution to be tested for inhibitory activity. The sodium deoxyribonucleate was prepared from calf thymus (Mirsky & Pollister, 1942) and served as substrate in the presence of veronal buffer and Mg ions (McCarty, 1946). The supernatant fluid of broth cultures of streptococci, strain C203S, with or without preliminary fractional precipitation with $(NH_4)_2SO_4$, was used as a source of deoxyribonuclease. There was no indication that the degree of inhibition was affected by the state of purity of the enzyme. In some experiments, deoxyribonucleases produced by other strains of streptococci (Wilson, group A; C7, group C) were employed in lieu of that formed by strain C203S.

RESULTS

Inhibition of deoxyribonuclease by crude bacterial extracts

Before attempting to isolate ribonucleic acid from bacterial and other sources, crude extracts of bacteria were prepared with a view to testing them for inhibitory action against group A streptococcal deoxyribonuclease.

(*a*) *Sonic extracts.* Neopeptone infusion broth (500 ml.) was inoculated with 0·5 ml. of broth culture and incubated at 37°. After 17 hr. the amount of growth was estimated by measuring against a broth blank the optical density of the culture in a Beckman model DU spectrophotometer (wavelength, 650 mμ.; light path, 10 mm.). The bacteria were

Table 1. *Inhibition of group A streptococcal deoxyribonuclease by extracts of various bacterial species*

Bacterial species	Method of disruption	Ratio of dry wt. of bacteria disrupted to vol. of extraction solution (mg./ml.)	Inhibition of group A streptococcal deoxyribonuclease (strain C203S) (%)	Inhibition of group A streptococcal deoxyribonuclease (strain Wilson) (%)	Inhibition of group C streptococcal deoxyribonuclease (strain C7) (%)
Escherichia coli (S3)	Sonic	5·8	88	64	6
Clostridium welchii (NYS)	Sonic	9·6	92	67	10
Pasteurella avicida (Hop)	Sonic	4·2	81	78	−2
Neisseria catarrhalis (Cor)	Sonic	1·2	41	33	−2
Bacillus subtilis	Sonic	2·1	72	67	4
Micrococcus pyogenes, var. *aureus* (209)	Sonic	5·4	77	72	−12
Corynebacterium xerose (NYS 155)	Sonic	1·9	10	−8	14
Escherichia coli (S3)	Ball mill	10	60	—	—
Clostridium welchii (NYS)	Ball mill	10	62	—	—
Pasteurella avicida (Hop)	Ball mill	7·2	54	—	—
Bacillus subtilis	Ball mill	10	63	—	—
Micrococcus pyogenes, var. *aureus* (209)	Ball mill	10	45	—	—
Corynebacterium xerose	Ball mill	9·2	29	—	—
Streptococcus pyogenes (C203S)	Ball mill	10	74	—	—

sedimented by centrifugation, the supernatant fluid discarded, and the bacteria washed in 250 ml. of saline buffered with phosphate at pH 7·0. The washed bacteria were suspended in 15 ml. of buffered saline and disrupted by exposing for 30 min. to the vibration of a 9000-cycle oscillator (Raytheon Corporation, Waltham, Massachusetts). The sonically treated material was centrifuged, and the supernatant fluid tested for inhibition by adding 0·2–0·4 ml. of deoxyribonuclease diluted in diluent (Bernheimer & Ruffier, 1951) to contain 30 units, and assaying the mixture. The inhibition produced by extracts of various bacterial species, tested against deoxyribonucleases formed by three streptococcal strains (C203S, group A; Wilson, group A; C7, group C), is shown in Table 1.

(*b*) *Extracts of ground bacteria.* The bacteria from 5 to 10 l. of a 17 hr. broth culture were washed in saline and freeze-dried. The dried bacteria, weighing about 1 g., were ground in a ball mill (Swift & Hirst, 1937; Bernheimer & Rodbart, 1948) for approximately 24 hr. and washed from the mill with small portions of cold distilled water totalling 100 ml. The pooled mill washings were centrifuged, and the clear supernatant fluid tested for inhibition of group A (strain C203S) deoxyribonuclease (Table 1).

The results show that sonic extracts of all bacterial species studied, with the exception of *Corynebacterium xerose*, partially inhibited the streptococcal deoxyribonuclease formed by group A streptococci (strains C 203S and Wilson) but failed to inhibit the deoxyribonuclease of the group C strain (C7). It is evident, therefore, that many bacterial species contain a substance which resembles streptococcal ribonucleic acid in its inhibitory effect on streptococcal deoxyribonucleases. The specificity of the inhibition suggests that the substance may be ribonucleic acid.

Extracts prepared from ground bacteria inhibited group A deoxyribonuclease, but were quantitatively somewhat less effective than the sonic extracts. It may be noted that the extract of ground *C. xerose* showed slight, but significant, inhibition as contrasted to the insignificant degree of inhibition shown by the sonic extract of the same organism.

In some instances, the inhibitory power of the extracts decreased during storage in the refrigerator for periods of a few days to a week. The loss appeared to be due to small amounts of bacterial ribonucleases, the presence of which was revealed by finding that some of the extracts were able to depolymerize commercial yeast nucleic acid.

Inhibition of deoxyribonuclease by purified ribonucleic acids

Ribonucleic acids were prepared from *Streptococcus pyogenes*, *Clostridium welchii*, *Escherichia coli*, wheat germ, beef liver and beef pancreas, according to the methods indicated in Table 2. The sources of additional preparations derived from tobacco leaves, tobacco mosaic virus and pancreas are also indicated in the table. The specific absorption at 260 mμ. and the phosphorus content of the preparations indicated that they had a purity of at least 50%. Some of them were known to be contaminated with deoxyribonucleic acid, but it is unlikely that the presence of this substance affected the results because authentic samples of deoxyribonucleic acid and its hydrolysis products failed to cause inhibition under the conditions of the test. In addition, all the preparations, including the crude extracts, were examined, with negative results, for the presence of contaminating deoxyribonuclease, which, if present, would have made the preparations unsuitable for the purpose at hand.

Table 2. *Capacity of various preparations of ribonucleic acids to inhibit group A streptococcal deoxyribonuclease*

Source of ribonucleic acid	Method of preparation	Concentration of ribonucleic acid preparation required to produce approx. 30% inhibition of deoxyribonuclease in test mixture (mg./ml.)
Liver (beef)	Mirsky & Pollister (1942) modified	>5·0
Liver (beef)*	Davidson & Waymouth (1944)	>5·0
Liver (beef)	Grinnan & Mosher (1951)	>5·0
Pancreas (beef)†	Johnson (unpublished observations)	>5·0
Pancreas (beef)*	Kerr & Seraidarian (1949)	>5·0
Wheat germ	Clarke & Schryver (1917) modified	>5·0
Yeast	Commerical	>5·0
Tobacco leaf‡	Pirie (1950)	>5·0
Tobacco mosaic virus, prep. 239G‡	Bawden & Pirie (1937)	5·0
Tobacco mosaic virus, prep. 339E‡	Bawden & Pirie (1937)	5·0
Tobacco mosaic virus, prep. 339D‡	Bawden & Pirie (1937)	>5·0
Tobacco mosaic virus, prep. 335A‡	Pirie (unpublished observations)	>5·0
Strep. pyogenes	Henry & Stacey (1946)	0·10
Cl. welchii	Henry & Stacey (1946)	0·23
Esch. coli	Sevag, Lackman & Smolens (1938)	0·31

* Prepared by Mr Clyde C. Doughty.

† We are greatly indebted to Dr Edward Johnson for supplying this preparation.

‡ For these nucleic acids we are greatly indebted to Mr N. W. Pirie who informs us that preparations 239G and 339E were isolated after splitting the virus nucleoprotein by the acetic acid method, while preparation 339D was isolated following fission by boiling and 335A by fission with $Sr(NO_3)_2$.

The ribonucleic acids were used in the form of their sodium or potassium salts. Each preparation was tested by mixing 0·2 ml. of solution containing 15 mg. ribonucleate/ml. with 0·4 ml. of group A streptococcal deoxyribonuclease containing approximately 75 units/ml., and assaying the mixture for depolymerase activity. If inhibition of more than 30% was observed, lower concentrations of ribonucleate were tested until the concentration producing approximately 30% inhibition was found.

The results (Table 2) show that the three ribonucleic acids of bacterial origin were effective in inhibiting the test deoxyribonuclease in relatively low concentration. In contrast, the ribonucleic acids derived from mammalian tissues as well as those obtained from yeast, wheat germ and tobacco leaves failed to inhibit the test enzyme in concentrations as high as 5 mg./ml. Several of the latter group of preparations tested at a level of 15 mg./ml. likewise failed to show significant inhibition. Two of the viral nucleic acids showed a qualitative resemblance to those of bacteria, but differed from them in the relatively high concentration needed to produce a significant degree of inhibition; the other two viral preparations did not inhibit in the concentrations tested.

Destruction of inhibitory activity by ribonuclease

It has been shown (Bernheimer & Ruffier, 1951) that the deoxyribonuclease-inhibiting property of a crude ribonucleate-containing streptococcal extract is destroyed by pancreatic ribonuclease. If the inhibition described in the previous section is due to ribonucleic acid, rather than to a contaminating substance in the preparations tested, it follows that the inhibitory power of the bacterial ribonucleic acids should be destroyed by treatment with ribonuclease. An experiment, the conditions and results of which are presented in Table 3, shows that the deoxyribonuclease inhibition is prevented by ribonuclease.

DISCUSSION

Recent studies (Chargaff *et al.* 1950) utilizing paper-chromatographic techniques have demonstrated differences in the quantities of purine and pyrimidine bases present in ribonucleic acids prepared from various animal and plant materials. There appear, however, to be no methods which permit differentiation of isolated ribonucleic acids without preliminary hydrolysis of the large molecules. A partial exception to this generalization depends upon the capacity of ribonucleic acid, or its ribonuclease-resistant fraction, to induce the formation of streptolysin S, ribonucleic acid derived from tobacco mosaic virus proving inactive in contrast to ribonucleic acids prepared from other sources, all of which were active in inducing streptolysin S formation (Bernheimer & Rodbart, 1948).

A second indication of individuality in unhydrolysed ribonucleic acids is provided by the data of the present report. It is shown that relatively low concentrations of bacterial ribonucleic acids partially inhibit group A streptococcal deoxyribonuclease, while the ribonucleic acids derived from mammalian tissues and from plants fail to exhibit a comparable effect even in concentrations 15–50 times greater than those of the bacterial preparations. The results are interpreted as pointing to the existence of at least two qualitatively different, naturally occurring types of ribonucleic acid.

Other interpretations of the findings can, however, be entertained. The methods used for the isolation of the bacterial ribonucleic acids differ from those employed for isolating ribonucleic acids from mammalian tissues, wheat germ, etc. and it is possible that the results reflect effects of the procedures used in extraction and purification rather than differences in the native nucleic acids. Attempts to test this possibility were made, but they failed because the methods used for the extraction of bacterial ribonucleic acids were found not to be

Table 3. *Prevention of deoxyribonuclease inhibition by pancreatic ribonuclease*

Solution tested for inhibitory activity*		Deoxyribonuclease found in test system (units/ml.)	Inhibition of deoxyribonuclease (%)
Deoxyribonuclease diluent (0·2 ml.)	+0·2 ml. water	57	0
Esch. coli ribonucleic acid (RNA) (0·4 mg. in 0·2 ml.)	+0·2 ml. water	25	60
Strep. pyogenes RNA (0·4 mg. in 0·2 ml.)	+0·2 ml. water	28	51
Esch. coli RNA (0·4 mg. in 0·2 ml.)	+2·0 μg. cryst. ribonuclease (0·2 ml.)	60	−5
Strep. pyogenes RNA (0·4 mg. in 0·2 ml.)	+2·0 μg. cryst. ribonuclease (0·2 ml.)	60	−5
Deoxyribonuclease diluent (0·2 ml.)	+2·0 μg. cryst. ribonuclease (0·2 ml.)	55	3

* The nucleic acids were dissolved in 'deoxyribonuclease diluent'; the ribonuclease in water. After 4 hr. at 37°, 0·2 ml. of each mixture was mixed with 0·4 ml. of a solution of partially purified group A streptococcal deoxyribonuclease diluted in 'deoxyribonuclease diluent' to contain approx. 75 units/ml. After standing a few minutes in an ice-water bath the mixtures were assayed for deoxyribonuclease. Controls not included in the table showed that ribonuclease, alone or mixed with ribonucleic acid, possessed no deoxyribonuclease activity.

applicable to tissue ribonucleic acid, and conversely. It may be noted, however, that a crude ribonucleate-containing extract of yeast was non-inhibitory (Bernheimer & Ruffier, 1951) even though prepared under the same conditions as those which yield inhibitory bacterial extracts.

It has been shown that the capacity of bacterial ribonucleic acids to inhibit streptococcal deoxyribonuclease is destroyed by minute amounts of pancreatic ribonuclease, and perhaps the non-inhibiting ribonucleic acids isolated from liver, pancreas, etc. have been altered by ribonuclease during the course of their extraction and purification. It seems probable that the testing of this hypothesis will have to await the discovery and application of effective inhibitors of ribonuclease. Likewise, it appears desirable to await the development of improved methods of isolation before attempting to extend the present observations to include ribonucleic acids of numerous other plant and animal species.

SUMMARY

1. A study has been made of the depolymerizing action of group A streptococcal deoxyribonuclease on thymus deoxyribonucleic acid as affected by ribonucleic acids of diverse origin. Crude and purified preparations of ribonucleic acids derived from a variety of bacteria partially inhibited the deoxyribonuclease. In contrast, ribonucleic acids obtained from mammalian pancreas and liver, tobacco leaves, wheat germ and yeast failed to show inhibition. Two preparations of tobacco mosaic virus nucleic acid resembled the bacterial ribonucleic acids in causing inhibition, but they were effective only in relatively high concentration.

2. The results are interpreted as indicating the possible existence in living matter of two qualitatively different types of ribonucleic acid: (*a*) an inhibitory form which is present in bacteria and possibly in some viruses, and (*b*) a non-inhibitory form which is present in mammalian tissue and in plants.

It is a pleasure to acknowledge the excellent technical assistance of Mrs Norma K. Ruffier and Miss Mary Elizabeth Farkas. It is a pleasure, too, to thank Mr N. W. Pirie, F.R.S., not only for supplying some of the nucleic acid preparations, but also for his continued interest in the work. This work was supported in part by grants from the Life Insurance Medical Research Fund and the Masonic Foundation for Medical Research and Human Welfare.

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A Biological Method of Assay of Hyaluronidase

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Hyaluronidase activity may be determined by various biological and physicochemical methods (Meyer, 1947) and theoretically any method measuring the depolymerizing activity of hyaluronidase may be considered suitable. The clinical efficacy of hyaluronidase, however, depends mainly on its power to split the highly polymerized mucopolysaccharides in the dermis, thus breaking down the barrier which hinders the inflow or the absorption of injected fluid. Hyaluronidase preparations, although purified during manufacture, may contain polysaccharidases which do not take part in the early stages of depolymerization. It is generally agreed that, if possible, the ultimate reference method of assay of hyaluronidase for therapeutic use should be a direct biological test based on de-

polymerization of the naturally occurring substrate and manifested by the spreading of an indicator; and that the results of any other method of assay should be closely correlated with those of the direct biological test. It is, however, widely considered that the spreading reaction cannot be used as an accurate assay of hyaluronidase and the correlation between a given biological method and the more commonly used physicochemical methods is rather poor (Meyer, 1947). Hechter (1950) has recently suggested that the spreading method would give reliable results provided certain conditions were observed. A reliable biological method of known precision was therefore sought which would make it possible to investigate the fitness of physicochemical methods for standardization of biological activity.

METHODS

Enzyme preparation. Seven preparations from different sources were used. Five of them (*A–E*) were of testicular origin in a more or less purified state. One had been prepared from staphylococci (*F*) and one (made by Dr J. H. Humphrey) from streptococci (*G*). Our laboratory standard preparation *A*, with which the other enzymes were compared, consisted of Seitz-filtered bull seminal fluid, distributed in equal amounts into ampoules, and dried from the frozen state. One ampoule contains approximately 50 mg. of the dry material. The other testicular enzymes were more or less purified commercially available preparations. They are listed in Table 1, which also shows widely different 'units' of activity in current use.

Solutions. The stock solutions of the enzymes were prepared in 0·9% (w/v) NaCl containing potassium phosphate buffer, pH 7·02, $I = 0·1$ with respect to the phosphate, and 0·2% (w/v) gelatin as stabilizer. They contained in addition 10 μg./ml. merthiolate. The pH of 7·02 was purposely chosen in order to simulate more closely physiological conditions (McClean, 1943), disregarding the fact that hyaluronidase is stated by Meyer (1947) to have a lower pH optimum. All dilutions were made with the above diluent. The stock solutions of 1·0 mg./ml. were kept at 4° and discarded after 3 weeks. During this time no appreciable loss of activity occurred as judged from the results of repeated bioassays and determinations of viscosity-reducing activity. Before use the dilutions were allowed to stand for about 1 hr. at room temperature.

Amounts injected. Volumes of 0·2 ml. were injected with an all-glass tuberculin syringe calibrated to 0·01 ml., through a short 26-gauge needle.

Indicator. Horse red cells were washed eight times with 0·9% (w/v) NaCl on the centrifuge in order to remove any serum inhibitor of hyaluronidase (McClean, 1942), then laked by the addition of distilled water, and the resulting solution was freeze-dried. From the dry material a 20% (w/v) solution was prepared in 0·9% (w/v) NaCl containing potassium phosphate buffer, pH 7·02, $I = 0·1$ and 0·2% (w/v) gelatin. Of this haemoglobin indicator solution 1 vol. was added to 4 vol. of enzyme dilution.

Animals. Twelve albino guinea pigs of 300 g. or over were used in each assay. The hair on their backs was mechanically clipped shortly before the assay, but the skin was not depilated chemically, in order to avoid possible skin lesions which might facilitate a leakage of the injected fluid.

Bioassay. The procedure finally adopted resembles the assay method as described by Humphrey (1943), but uses freshly killed animals as recommended by Hechter (1947) in order to avoid spread of the indicator dye by extravasation of fluid from the capillaries which may occur in the skin of living animals. It also makes use of a standard preparation with which the other enzymes are compared.

In preliminary tests using the whole back of the animal a 16-point assay with latin square design was carried out. Using four twofold dilutions of enzyme, the highest of which produced about 20% increase of the mean area compared with the control, it was ascertained that, within the dose range employed, the departure from linearity of the curves of response (area of spread of indicator) plotted against log dose, was not significant. In these assays, however, the site to site variations were excessive and the lesions tended to spread into one another. It was therefore decided to use a skin area in which such variations could be reduced to a minimum. This area is limited cranially by the interscapular pad of fat, posteriorly by the pelvis and extends ventrally on both sides to a line parallel to and 1 in. distant from the midline. Within this area two dilutions of the standard preparation (4 and 2 μg./ml.) and two appropriate twofold dilutions of the enzyme under test, that is a total of four injections, were distributed at random. The dilutions of the test preparation were made as nearly as possible equipotent with the dilutions of the standard. The time elapsing between the animal's death, by a blow on the head and bleeding from the neck, and the start of the first injection was usually not more than 1 min.; subsequent injections were made at 15 sec. intervals. 10·5–11 min. after the first injection the skin of the back was flayed, care being taken neither to pull on the skin nor to press on the blebs. The skin, with the inside surface uppermost, was laid beneath a flat glass plate and the outline of each bleb traced on cellophan exactly 12 min. after the injection was made. These tracings were copied on parchment paper and the resulting areas determined by cutting out and weighing the pieces of parchment. The time of 12 min. chosen for the reading is much shorter than in most of the biological methods in use (Duran-Reynals, 1946; Bacharach, Chance & Middleton, 1940); it is intermediate between that recommended by Hechter (1950) and the reaction time used by Humphrey (1943). At this time the blebs are still actively diffusing with approximately their initial velocity.

RESULTS

Since two doses of the standard and two of the test enzyme were administered, the assay represents a 4-point design. The estimation of potency and the statistical analysis were performed accordingly, including an analysis of variance and a test for parallelism of the dose-response lines. Table 1 gives the mean potencies of the different enzymes tested in the terms of the standard and their respective limits of error. Each value has been computed from two to three assays. It can be seen that three of the partially purified testicular enzymes were several times more potent than the laboratory standard preparation which consisted simply of filtered and freeze-dried bull seminal fluid, whereas the fifth

Table 1. *The activity of various hyaluronidase preparations compared by bioassay*

Hyaluronidase preparation	Stated activity	Bioassay activity in terms of standard preparation	Limits of error (%)
A standard	—	1	—
Testicular enzymes:			
B	1000 manufacturer's units/ampoule (3·5 mg.)	5·1	75·3–132·8
C	Spreading activity when injected intradermally at dilutions of 1:50 000 or more into the skin of guinea pigs	5·4	82·5–121·3
D	152 turbidity-reducing 'units'/mg. ± 14	6·8	85·8–116·5
E	150 turbidity-reducing 'units'/vial (3·2 mg.)	0·59	70·6–124·9
Bacterial enzymes:			
F	—	2·3	78·8–126·9
*G**	—	0·54	85·6–116·9

* The dry preparation contained 85 % gum arabic as a stabilizing agent. The actual activity of the hyaluronidase was therefore 3·6 × that of the standard preparation.

testicular enzyme preparation was definitely less potent. Since the standard enzyme was consistently used in concentrations of 4 μg./ml. and 2 μg./ml. this implies that the more potent members of this series were still remarkably active in concentrations of about 0·3 μg./ml.

DISCUSSION

With the precautions outlined above satisfactory skin-diffusion assays were achieved, but only when using concentrations of the order of 1 μg./ml. of ordinary commercially available preparations. This consideration throws some doubt on whether experiments performed with far higher concentrations of relatively pure enzymes really measure disintegration of the native substrate, and not a non-specific break-down of other barriers such as the complex of the capillary wall, thus involving capillary permeability rather than dermal tissue permeability. In experiments employing prolonged reaction times and concentrations of hyaluronidase far in excess of those required to show an effect, results have been obtained by other workers which have been taken to indicate an antagonistic action of adrenocorticotrophic hormone, adrenocortical steroids, salicylates etc. These results need not, however, be interpreted as demonstrating a direct inhibitory action of such compounds on hyaluronidase. This has been clearly pointed out by Hechter (1950), who also argued that the early phase of the spreading reaction after hyaluronidase administration is the only stage which can be regarded as exclusively due to hyaluronidase.

Using the biological assay method described, tests which were performed 2 months apart showed reproducible and consistent estimates of potency. By the use of twelve animals per assay and choice of those skin sites which showed the smallest variation, the errors of the method have been reduced to a tolerable minimum. The statistical analysis showed that the average limits of error were less than 30 %, thus making it possible to distinguish easily between twofold differences in activity.

In view of the number of variables which may affect the biological hyaluronidase assay and of the difficulty in controlling them, it is clear that the potency of a given hyaluronidase preparation must be defined in terms of a standard preparation. In a subsequent paper the results of estimates by the bioassay will be compared with those by the more usual physicochemical methods.

SUMMARY

1. A method of biological assay of hyaluronidase is described in which use is made of the early spreading activity of highly diluted hyaluronidase preparation in the skin of freshly killed guinea pigs, and the activity compared with that of a standard preparation.

2. When twelve animals per assay and a properly randomized arrangement of the injections were employed, the estimates of potency had fiducial limits ($P < 0{\cdot}05$) ranging from ± 16 % to ± 32 %.

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Hyaluronidase: Correlation between Biological Assay and other Methods of Assay

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Different workers have at various times sought to correlate the *in vivo* and *in vitro* activities of hyaluronidase preparations. Chain & Duthie (1940), drawing attention to the association between diffusion in the tissues and the mucolytic activity of testicular extracts, pointed out that the viscosity-reducing and diffusing activities of various enzyme preparations were in good agreement. McClean (1943) compared enzymes from different sources by their diffusing activity with Humphrey's (1943) technique, by their viscosity-reducing activity at pH 7·0 and by the mucin-clot prevention test; he found that the results of the viscosimetric assay could be very closely correlated to those of the skin-diffusing activity. Madinaveitia, Todd, Bacharach & Chance (1940), on the other hand, were unable to correlate satisfactorily the viscosity-reducing activity of hyaluronidase preparation with their skin-diffusing activity and stated that diffusing factors ought not to be assayed in terms of their activity in the physicochemical test. In his review on the biological role of hyaluronic acid and hyaluronidase, Meyer (1947) pointed out that the spreading reaction could not be considered as an accurate assay of hyaluronidase and that the correlation between spreading reaction and physicochemical methods of hyaluronidase assay was poor. In certain instances the comparison was vitiated by the use of different conditions of pH and salt concentration in the different tests, but even when this was avoided the error of the biological assays was too great to permit any conclusion that *in vitro* tests are a true measure of *in vivo* activity. The question is of some importance because of the need to standardize hyaluronidase in view of its increasing use in clinical medicine. Having devised a biological method of hyaluronidase assay which gives reproducible results (Jaques, 1953) we undertook to assay seven different enzyme preparations by the more convenient viscosimetric and turbidimetric methods and to correlate the results with those of the bioassay. All tests were performed under approximately physiological conditions of pH and salt concentration in order to imitate more closely the conditions of the skin test (McClean, 1941, 1943).

METHODS

Substrates. Two different hyaluronate preparations were used. (*a*) A commercial potassium hyaluronate (Allen and Hanbury) containing approximately 60% hyaluronate (measured as glucosamine) dissolved in 0·9% (w/v) NaCl containing potassium phosphate buffer, pH 7·0, $I = 0·1$ with respect to phosphate, and 1:100 000 thiomersalate. This preparation will be referred to below as 'commercial hyaluronate'; it was used at 0·2 and 0·35% (w/v) in viscosimetric and turbidimetric assays respectively. (*b*) Purified potassium hyaluronate prepared by Dr H. J. Rogers from umbilical cords by the method of Hadidian & Pirie (1948). This preparation contained no N other than glucosamine N. It will be referred to as 'purified hyaluronate'. It was used, in the same solvent, at 0·1 and 0·2% (w/v) in viscosimetric and turbidimetric assays respectively.

The two batches of potassium hyaluronate were employed for both the viscosimetric and turbidimetric assays of all seven enzyme preparations. In the light of the observations of Meyer (1951) that various metals, notably Fe, are potent inhibitors of hyaluronidase, many of the assays with gelatin as stabilizer and commercial hyaluronate as substrate were repeated in the presence of 0·2% (w/v) sodium pyrophosphate; this substance was added in all assays with the purified substrate, although with our reagents it did not appear to have any appreciable effect.

Enzyme solutions. These were made in 0·9% (w/v) NaCl containing phosphate buffer, pH 7·02, $I = 0·1$ with respect to phosphate, containing 0·2% (w/v) gelatin and 1:100 000 thiomersalate. Stock solutions containing 1–10 mg. enzyme/ml. lost no appreciable activity during 3 weeks' storage at 4°.

Biological assay. The procedure was that described by Jaques (1953).

Viscosimetric method. The method of McClean & Hale (1941) was used.

Viscosimeters. The viscosimeters employed were of an Ostwald type calibrated in absolute viscosity units over a range of viscosities studied. (Supplied by Dr L. A. Steiner, 76 Cavendish Road, London, S.W. 12.) Flow time with water at 30° was of the order of 65 sec. The water bath in which the viscosimeters were immersed was kept at 30±1°. The general conduct of the tests followed the lines suggested by McClean & Hale (1941) who defined a viscosity-reducing unit as the amount of enzyme which will reduce the viscosity of the substrate in 20 min. to a level half-way between its original figure and that of the solvent employed. Our results were calculated on this basis.

Turbidimetric method. The method used was based upon that of Tolksdorf, McCready, McCullagh & Schwenk (1949) in which the unit of activity is taken as the quantity of enzyme required to reduce the turbidity given by 0·2 mg. of purified hyaluronate to that given by 0·1 mg. when incubated together for 30 min. at 37° in 0·1 M-acetate buffer, pH 6·0, containing 0·15 M-NaCl. The turbidity is developed, after arresting the enzyme action by heating for 10 min. at 60°, by addition of a 1:10 dilution of serum or plasma previously heated at pH 3·1, and an excess of acetate buffer, pH 4·2.

Since we wished to make comparison with other assays performed at physiological pH and salt concentrations, and to guard against inactivation of hyaluronidase which occurs spontaneously in high dilutions in the absence of other colloids, the method was modified. The buffered saline described above was used throughout, and gelatin or, in some cases, gum acacia was added to give concentrations of 0·1 or 0·25% respectively in the enzyme-substrate mixtures. The period of incubation at 37° was shortened to 10 min., since evidence was obtained that the rate of enzyme action diminished quite markedly with further incubation. Furthermore, the reaction was stopped by chilling at 0°, followed by addition of dilute acidified horse serum, rather than by heating at 60° which permits considerable further enzyme action to take place.

RESULTS

Table 1 compares the results of the three different methods of assay, the potency of the standard enzyme, which was always assayed simultaneously, being taken as unity. At least two assays, and often many more, were performed with each enzyme by each method. The potencies of the various enzymes as determined with the viscosimetric method are in reasonable agreement with those of the bioassay. In the viscosimetric assay the nature of the substrate apparently did not affect the estimation of potency of the majority of enzymes tested, the only marked difference occurring with the streptococcal enzyme (*G*) which was less active upon the purified substrate. With the pure hyaluronate the value for *G* corresponds much better with

Table 1. *Comparison of the activity of several hyaluronidase preparations using various assay methods*

Enzymes	Bioassay activity in terms of standard (b)	Viscosimetric method activity in terms of standard: Commercial hyaluronate (v_1)	Viscosimetric method activity in terms of standard: Purified hyaluronate (v_2)	Turbidimetric method activity in terms of standard: Commercial hyaluronate (t_1)	Turbidimetric method activity in terms of standard: Purified hyaluronate (t_2)	Ratios: b/v_1	b/v_2	b/t_1	b/t_2
A, standard; testis	1	1	1	1	1	1	1	1	1
B, testis	5·1	4·5	5·4	2·1	2·2	1·1	0·9	2·4	2·3
C, testis	5·4	6·6	6·9	2·6	2·3	0·8	0·8	2·1	2·3
D, testis	6·8	7·6	7·2	3·8	2·6	0·9	0·95	1·8	2·6
E, testis	0·59	1·0	0·61	0·5	0·38	0·6	1·0	1·2	1·5
F, *Staphylococcus*	2·3	2·6	1·7	0·8	0·54	0·9	1·4	2·9	4·3
G, *Streptococcus*	0·53	1·4	0·65	0·2	0·16	0·4	0·8	2·7	3·4

Table 2. *The potency of several hyaluronidase preparations*

Enzymes	Concentration of enzyme which reduced the viscosity of the substrate by half in 20 min. at 30° (μg./ml.): Substate commercial potassium hyaluronate (0·2 mg./ml.)	Concentration of enzyme which reduced the viscosity of the substrate by half in 20 min. at 30° (μg./ml.): Substrate purified potassium hyaluronate (0·1 mg./ml.)	Concentration of enzyme which reduced the turbidity value of the substrate by half in 10 min. at 37° (μg./ml.): Substrate commercial potassium hyaluronate (0·35 mg./ml.)	Concentration of enzyme which reduced the turbidity value of the substrate by half in 10 min. at 37° (μg./ml.): Substrate purified potassium hyaluronate (0·2 mg./ml.)
A, standard	5·5	7·03	71	91
B	1·19	1·3	34·5	41·6
C	0·83	1·01	27	40
D	0·73	0·98	19·2	35
E	5·4	11·5	133	240
F	2·04	4·13	91	167
G	4·05	10·9	370	570

that obtained in the bioassay. The overall agreement of biological and viscosimetric assay is brought out in the column wherein are listed the ratios of both assays with each substrate. On the other hand, the correlation between the turbidimetric results and this bioassay is less good. Thus, in the biological assay the preparations *B* and *C* were both about five times as potent as the standard, whereas turbidimetrically their activities in terms of the standard were respectively 2·1 and 2·6. With the bacterial enzymes *F* and *G* the discrepancy between the results of both methods was even more pronounced. In Table 2 some experimental values are given, expressed as reciprocal dilutions (w/v) of the various enzyme preparations required to reduce the viscosity due to the substrate by half in 20 min., or the turbidity by half in 10 min., under the conditions outlined above.

When 0·2 % (w/v) gelatin was used as stabilizer in the diluting fluid, the shape of the curves of turbidity (absorptiometer reading), after the set period of incubation, against quantity of enzyme was closely similar for all the enzyme preparations, and the relative potencies calculated in terms of any arbitrary turbidity end point were likewise similar. In earlier experiments 0·5 % (w/v) gum acacia was used instead of gelatin, and it was regularly observed that preparations *A*, *B* and *D* gave curves which had a steeper slope than those given by preparations *C* and *G*. The relative potencies in the gum acacia experiments were therefore dependent upon the turbidity end point chosen, and consequently also upon the time taken to reach this end point.

Table 3. *Relative activities of enzyme preparations per mg. compared with standard preparation by the turbidimetric method, using a single preparation of the substrate*

(Method (*a*): incubated 10 min. in presence of 0·2% (w/v) gelatin. Method (*b*): incubated 30 min. in presence of 0·5% (w/v) gum acacia.)

Enzyme preparation	Method (*a*)	Method (*b*)
B	2·1	1·26
C	2·6	2·5
D	3·8	1·4
G	0·2	0·27

The importance of this effect is shown in Table 3, where the apparent activities of four preparations upon the same commercial hyaluronate substrate compared with the activity of the standard are shown (*a*) measured in presence of 0·2 % (w/v) gelatin with 10 min. incubation and (*b*) measured in presence of 0·5 % (w/v) gum acacia with 30 min. incubation. Whatever the cause of the discrepancies in the presence of gum acacia, it appears to be unsuitable for use as a stabilizing agent in this test.

DISCUSSION

The possession of a reasonably accurate biological assay method for hyaluronidase has made it possible to test the validity of the correlation between activity *in vivo* and *in vitro*, measured by viscosimetric and turbidimetric methods. We have shown that, provided suitable precautions are taken, among them the use of a reference standard enzyme preparation in each test, the correlation for the viscosimetric method is good and nearly as good for the turbidimetric method. If the bacterial enzymes are excluded, which is probably justified since the reference standard was a testicular preparation, it may be deduced that the viscosimetric assay gave an accurate measure of the biological activity, especially when the purified substrate was used. The turbidimetric assays all appear to have given relatively low values compared with the bioassay. Inspection of the figures in Table 1, however, suggested that if one of the purified testicular enzymes had been used as the reference standard, the results of the assays by all three methods would have shown ratios close to unity, at least in so far as other purified testicular preparations were concerned. The figures were therefore recalculated on the basis of unit potency for preparation *C*, and in Table 4 are shown the ratios obtained. The agreement between the results of the biological and the other assays is very good for the purified testicular preparations, and it seems likely that for use as a practical biological standard, valid for all three methods, a partly purified testicular preparation would be preferable to crude seminal fluid, although the latter was originally chosen with the idea that it would contain any enzyme or enzymes to be found in the purer preparations.

The fact that the correlation is less good with the two bacterial enzymes is not surprising, since there is no evidence that they are single enzymes or that their action is identical with that of testicular preparations.

The influence of the nature of the substrate upon the results of *in vitro* methods of hyaluronidase assay has been mentioned by many workers. We began this work with the expectation that if a reference preparation of enzyme were used in all assays no influence of the substrate would be apparent, since all like enzyme preparations, at equivalent concentrations of active material, should be similarly affected by variations in degree of polymerization, content of sulphated polysaccharide or presence of enzyme inhibitors. This expectation was not fulfilled, and the apparent variations in activity of the enzymes with the pure and the cruder substrate were outside the range of experimental error. Furthermore, the effect of using gum acacia as stabilizer instead of gelatin was

Table 4. *Ratio of activities by different assay methods, when activity of preparation C is taken as* 1

Enzyme source	Biological assay Viscosimetric assay		Biological assay Turbidimetric assay	
	Commercial substrate	Pure substrate	Commercial substrate	Pure substrate
A, seminal fluid	1·26	1·26	0·5	0·45
B, testis	1·38	1·22	1·17	1·0
C, testis	1	1	1	1
D, testis	1·1	1·2	0·87	1·1
E, testis	0·75	1·2	0·58	0·69
F, *Staphylococcus*	0·91	0·58	0·72	0·54
G, *Streptococcus*	2·1	0·9	0·8	0·7

markedly different upon different enzyme preparations. These observations imply some degree of heterogeneity of the enzyme preparations compared. It is of interest, from a practical point of view, that the closest agreement between *in vitro* and *in vivo* activity was obtained with the purest substrate.

SUMMARY

1. Five preparations of testicular hyaluronidase, one of streptococcal and one of staphylococcal origin were compared by an accurate skin-diffusion assay and by viscosimetric and turbidimetric methods.

2. With testicular preparations the results of the three methods agree well, provided that the potency is measured in terms of a reference preparation of enzyme, and that the pH and ionic strength of the solvents are approximately physiological. It is desirable to use highly purified substrate, and to stabilize the enzymes with gelatin rather than gum acacia.

3. The activities of the two bacterial enzymes showed a close but not complete correlation between the three tests.

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The Differentiation of True and Pseudo Cholinesterase by Organo-phosphorus Compounds

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True cholinesterase is the enzyme present in the brain and erythrocytes of many species which hydrolyses acetylcholine at a higher rate than butyrylcholine and is inhibited by excess substrate. Pseudo cholinesterase is present in the serum of many species and hydrolyses butyrylcholine at a higher rate than acetylcholine and is not inhibited by excess substrate. Mendel, Mundell & Rudney (1943) introduced the substrates acetyl β-methylcholine and benzoylcholine for true and pseudo cholinesterases respectively. Differentiation of true and pseudo cholinesterases has also been achieved by the use of selective inhibitors. Di*iso*propyl fluorophosphonate (DFP) inhibits pseudo cholinesterase at a lower concentration than the true (Mazur & Bodansky, 1946; Mendel & Hawkins, 1947; Adams & Thompson, 1948). However, in some species the sensitivities of the two enzymes are not very different (Ord & Thompson, 1950). A series of organo-phosphorus compounds have been ex-

amined for their differential inhibitory power. Some of these compounds are superior, in some respects, to DFP for this purpose.

Evidence has been produced which suggests that organo-phosphorus inhibitors are substrates for cholinesterase and are hydrolysed (Aldridge & Davison, 1952*a*, *b*), but that one of the products, the substituted phosphoric acid, remains attached to the enzyme (Boursnell & Webb, 1949; Michel & Krop, 1951). The most outstanding difference between true and pseudo cholinesterases is their substrate specificity for acetates and butyrates respectively (Adams, 1949; Adams & Whittaker, 1949; Sturge & Whittaker, 1950). If the mechanism of inhibition of the true and pseudo cholinesterases by organo-phosphorus inhibitors is the same and they are attached to the ester-attracting site by the electrophilic phosphorus atom (Nachmansohn & Wilson, 1951) then the length of the alkoxy group attached to the phosphorus may well be important in governing the 'fit' of the inhibitor on the enzyme surface and thus its ease of hydrolysis and inhibitory power. A comparison has been made between the substrate and inhibitor specificity of true and pseudo cholinesterases of horse blood. The mechanism suggested above would explain the results we have obtained on the comparative inhibition of true and pseudo cholinesterases of horse blood by various organo-phosphorus inhibitors.

METHODS

Cholinesterase has been determined manometrically as described in detail previously (Aldridge & Davison, 1952*a*). For inhibitor experiments whole horse blood has been used as the source of true and pseudo cholinesterases with acetyl β-methylcholine as substrate for cholinesterase in the erythrocytes and benzoylcholine for pseudo cholinesterase of the serum. A measurable output of CO_2 is produced with both substrates from 0·5 ml. defibrinated whole blood. For the determination of the substrate specificity horse serum was used for pseudo cholinesterase and a partially purified preparation of horse erythrocyte stroma for true cholinesterase. The purified stroma was prepared as follows: defibrinated horse blood (1200 ml.) was washed twice with 2% w/v NaCl (saline) solution. It was centrifuged and resuspended to 1 l. in saline and poured rapidly into 1 l. 0·2% (w/v) $BeSO_4 . 4H_2O$ in saline. This causes the cells to agglutinate. It was then diluted to 10 l. with saline and the supernatant removed when the cells had settled. The washing procedure was repeated and tap water then run in up to 10 l. It was left 45 min. for the cells to haemolyse. 1 l. 20% (w/v) NaCl solution was added. The supernatant was removed when the cells had settled and the washing with saline repeated until the top layer was only pale red (five times). After centrifuging at +1°, the pale red stroma (approx. 100 ml.) was collected. Sodium citrate (20 g./100 ml. stroma) was added and left overnight at −5°. Four times its volume of 27% (v/v) mixture of ethanol and water was run in slowly at −5° stirring all the time. After standing 1 hr. it was centrifuged at −5°. The resulting buff-coloured precipitate has an activity against acetylcholine of 1·32 μl. CO_2/mg. dry wt./min., a 16-fold purification over the original packed cells.

The choline esters used for the determination of substrate specificity were prepared by heating 1·2 mol. of the corresponding acyl chloride with 1 mol. choline perchlorate for 1 hr. at 140–150°. The resulting choline ester perchlorates were recrystallized from dry *iso*propanol. All gave a positive reaction for esters by Hestrin's (1949) reaction and have the following melting points: acetyl 114°, propionyl 103–105°, *n*-butyryl 72°, *iso*butyryl 117°, and *iso*valeryl 35°. The *n*-valerylcholine iodide was kindly supplied by Mr D. R. Davies (Chemical Defence Experimental Establishment, Porton), the benzoylcholine chloride was from Roche Products and acetyl β-methylcholine bromide from L. Light and Co., Ltd. All substrates were used at a final concentration of 0·015 M. The perchlorate ion has no effect on the activity of the enzyme since true cholinesterase (horse erythrocytes) has the same activity against acetylcholine chloride and perchlorate and pseudo cholinesterase (horse serum) against *n*-butyrylcholine chloride and perchlorate.

In all inhibitor experiments the enzyme has been incubated with the inhibitor for 30 min. at 37° prior to the addition of substrate. Inhibitor solutions in buffer ($NaHCO_3$ 0·0357 M; $MgCl_2$, 0·040 M; NaCl, 0·164 M) have been prepared fresh for every experiment. The concentration of inhibitor necessary to produce 50% inhibition of enzyme activity under these conditions has been determined by plotting $\log_{10}$ residual activity against M concentration of inhibitor. This inhibitory power has usually been expressed as $pI_{50} = -\log$ M concentration of inhibitor to produce 50% inhibition. The rate of hydrolysis of choline esters by pseudo cholinesterase divided by that for true cholinesterase is called the substrate ratio. The concentration of inhibitor necessary to produce 50% inhibition of true cholinesterases divided by that for pseudo cholinesterase is called the inhibitor ratio.

RESULTS

Comparison of the sensitivities of true and pseudo cholinesterase to organo-phosphorus compounds. It has been demonstrated that di*iso*propyl fluorophosphonate (Mazur, 1946) tetraethyl pyrophosphate (Aldridge & Davison, 1952*a*), and diethyl *p*-nitrophenyl phosphate (Aldridge, 1953*a*) are hydrolysed by the tissues of various mammalian species. A comparison of the sensitivity of true and pseudo cholinesterases, in an unpurified condition, to such organo-phosphorus inhibitors is difficult to assess. The results in Table 1 show that with diethyl *p*-nitrophenyl phosphate (E 600) as inhibitor less inhibitor is required to inhibit erythrocyte cholinesterase in the absence than in the presence of serum. This is due to the presence of an enzyme hydrolysing E 600 in horse serum (Aldridge, 1953*a*). Two other compounds examined in this way, bismono*iso*propylamino fluorophosphine oxide (Isopestox) and di*iso*propyl fluorophosphonate (DFP), show little difference in behaviour with or without horse serum.

Both the cholinesterases of whole horse blood may be treated with an inhibitor under the same

conditions and the effect on true and pseudo enzymes separately determined using the specific substrates, acetyl β-methylcholine and benzoylcholine (Mendel *et al.* 1943). By this means more comparable estimates of the sensitivities of the two enzymes to different inhibitors may be obtained. With some inhibitors the concentration necessary for 50% inhibition of true cholinesterase is much higher than that for pseudo cholinesterase. In this case, although the same amount of enzyme which may destroy the inhibitor is present in the two cases, it is presented with different concentrations of inhibitor. Experimental evidence of the kinetics of hydrolysis of substrates at concentrations as low as 10^{-8} M is extremely limited, but it is probable that the hydrolysis will follow first-order kinetics (Van Slyke, 1942); with a constant enzyme concentration and time of incubation (for the experiment) a constant proportion of the inhibitor will be removed. It is, therefore, unlikely that an appreciable error will be produced from this source.

The results obtained for various inhibitors using the above technique are given in Table 2. Two inhibitors show a high inhibitor ratio, Isopestox and tetra*iso*propyl pyrophosphoramide (ISO-OMPA). Both of these compounds are crystalline, practically non-volatile, soluble in water and are more

Table 1. *The effect of horse serum on the inhibition of horse-erythrocyte cholinesterase by various inhibitors*

(Defibrinated horse blood or washed horse erythrocytes (0·5 ml.) incubated for 30 min. at 37° with various concentrations of inhibitor. After 30 min. the acetyl β-methylcholine was tipped in and the enzyme activity remaining determined, $pI_{50} = -\log_{10}$ M concentration producing 50% inhibition after incubation for 30 min. at 37°.)

Inhibitor	pI_{50} Whole horse blood	pI_{50} Washed horse erythrocytes
Diethyl *p*-nitrophenyl phosphate (E 600)	6·31	7·33
Bismono*iso*propylamino fluorophosphine oxide (Isopestox)	3·77	3·77
Di*iso*propyl fluorophosphonate (DFP)	5·70	5·68

Table 2. *The inhibition of true and pseudo cholinesterases of horse blood by various inhibitors*

(Defibrinated horse blood (0·5 ml.) incubated for 30 min. at 37° with various concentrations of inhibitor. After 30 min. the substrates were tipped in and the remaining enzyme activity determined. Acetyl β-methylcholine was used to determine erythrocyte cholinesterase and benzoylcholine to determine the serum enzyme. $pI_{50} = -\log_{10}$ M concentration of inhibitor to produce 50% inhibition. ChE = cholinesterase.)

Inhibitor	pI_{50} Pseudo ChE	pI_{50} True ChE	Inhibitor ratio (True ChE/pseudo ChE)
Dimethyl *p*-nitrophenyl phosphate	6·10	6·27	0·67
Diethyl *p*-nitrophenyl phosphate (E 600)	6·85	6·38	2·9
Di*iso*propyl *p*-nitrophenyl phosphate	6·50	5·50	10
Tetraethyl pyrophosphate (TEPP)	8·39	6·52	73
Tetra*iso*propyl pyrophosphate	8·68	5·96	520
Di*iso*propyl fluorophosphonate (DFP)	8·18	5·75	270
Diethyl fluorophosphonate	7·80	6·09	52
Bisdimethylamino fluorophosphine oxide	3·31	1·96	22
Mono*iso*propylamino dimethylamino fluorophosphine oxide	5·10	3·42	48
Bismono*iso*propylamino fluorophosphine oxide (Isopestox)	7·42	3·82	3950
Tetra*iso*propyl pyrophosphoramide (ISO-OMPA)	6·48	2·51	9400

Table 3. *The sensitivity of the true and pseudo cholinesterases of different species to tetraisopropyl pyrophosphoramide and bismonoisopropylamino fluorophosphine oxide*

(Erythrocytes washed three times with saline were used as a source of true ChE and plasma or serum for pseudo ChE except in the case of the rat where rat-brain and rat-heart homogenates were used respectively. The enzyme was incubated with inhibitor for 30 min. at 37° prior to the addition of substrate. Acetylcholine was the substrate except for rat heart when benzoylcholine was used. $pI_{50} = -\log_{10}$ M concentration of inhibitor which will produce 50% inhibition. ChE = cholinesterase.)

Species	ISO-OMPA pI_{50} True ChE	ISO-OMPA pI_{50} Pseudo ChE	ISO-OMPA Inhibitor ratio	Isopestox pI_{50} True ChE	Isopestox pI_{50} Pseudo ChE	Isopestox Inhibitor ratio
Horse	2·47	6·52	11 300	3·80	7·42	4 200
Human	3·52	5·27	56	4·66*	6·41*	56
Guinea pig	2·34	5·89	3 600	4·21	6·51	200
Dog	2·42	6·54	13 200	4·06	7·29	1 700
Rat	3·60	6·32	530	4·22	6·75	340
Sheep	3·20	—	—	4·41	—	—

* These results are in confirmation of an observation by Austin & Berry, 1952.

stable to hydrolysis than DFP. They are, therefore, more convenient to handle. The inhibitor ratio for DFP for some species is particularly low (Ord & Thompson, 1950). The results in Table 3 show that Isopestox and tetra*iso*propyl pyrophosphoramide also show species differences and care must also be used to differentiate true and pseudo cholinesterase with these inhibitors.

Substrate specificity of true and pseudo cholinesterase of horse blood. It is clear from the work of Whittaker (1951) that the optimum acyl group of substrates for true cholinesterase is acetyl and, for pseudo cholinesterase, butyryl. To examine the possibility that length of the alkyl chain from the phosphorus atom governed the specificity of the inhibitors it was necessary to determine the substrate specificity for a series of choline esters. For instance, calculation from covalent single-bond radii of carbon, oxygen and phosphorus atoms (Remick, 1949) shows that the length of C—C—C chain of propionylcholine is 3·08 A. while that for P—O—C chain in dimethyl *p*-nitrophenyl phosphate is 3·19 A. The lengths of the alkyl chains in these two compounds are approximately the same. On this basis the length of the alkyl chain in di*iso*propyl fluorophosphonate will be equivalent to that for *iso*valerylcholine. A series of choline esters have therefore been synthesized and their hydrolysis by erythrocyte and serum cholinesterase of horse blood has been determined. The results are given in Table 4. Up to *n*-butyrylcholine our results agree with those of Glick (1941) for serum cholinesterase and of Mounter & Whittaker (1950) for erythrocyte cholinesterase. Chain length as well as side-branching of the acyl group have a profound effect on the rates of hydrolysis of choline esters. A comparison of the rates of hydrolysis of a choline ester by the two enzymes is given by the substrate ratio.

Mechanism of the differentiation of true and pseudo cholinesterase by organo-phosphorus compounds. When chymotrypsin is inhibited by DFP there is evidence that the inhibitor is hydrolysed and the dialkyl phosphate group remains attached to the enzyme (Jansen, Nutting & Balls, 1949; Jansen, Nutting, Jang & Balls, 1950). This has been extended to the inhibitor E 600 (Hartley & Kilby, 1952). If cholinesterase is inhibited by a similar mechanism, and there is indirect evidence that this is so (Aldridge & Davison, 1952*a*; Boursnell & Webb, 1949), then the inhibitor is acting as a substrate and being

Table 4. *Hydrolysis of choline esters by horse-erythrocyte and serum cholinesterases*

(Horse serum was used directly. The erythrocyte cholinesterase was a purified stroma preparation as described under Methods. All substrate concentrations were 0·015 M. Results are corrected for non-enzymic hydrolysis. No output of CO_2 was obtained without the addition of substrate. Readings were taken every 5 min. except for *iso*valerylcholine with true ChE (30 min.). Activities are all based on acetylcholine as 100. ChE = cholinesterase.)

Choline ester	Pseudo ChE	True ChE	Substrate ratio (pseudo ChE/true ChE)
Acetyl	100	100	1·0
Propionyl	145	78	1·9
n-Butyryl	216	2·3	94
*iso*Butyryl	91	46	2·0
n-Valeryl	145	3·3	44
*iso*Valeryl	50	0·19	263

Table 5. *A comparison of the substrate ratio and the inhibitor ratio for true and pseudo cholinesterase of the horse*

(Substrate ratios and inhibitor ratios are as given in Tables 4 and 2 respectively.)

Substrates		Inhibitors	Inhibitor ratios			
Acyl group of choline ester	Substrate ratio	Alkyl group of inhibitor	*p*-Nitrophenyl phosphates	Fluoro-phosphonates	Pyro-phosphates	Fluorophos-phoramide
$-CO.CH_3$	1	$>PO.CH_3$	—	—	—	—
$-CO.CH_2.CH_3$	1·9	$>PO.OCH_3$	0·68*	—	—	—
$-CO.CH_2.CH_2.CH_3$	94	$>PO.OCH_2CH_3$	2·9	51	75	—
$-CO.CH(CH_3)_2$	2·0	$>PO.N(CH_3)_2$	—	—	—	22
$-CO.CH_2.CH_2.CH_2.CH_3$	44	$>PO.OCH_2.CH_2.CH_3$	—	—	—	—
$-CO.CH_2.CH(CH_3)_2$	263	$>PO.OCH(CH_3)_2$	10	270	500	3950

* We have evidence that the inhibition by dimethyl *p*-nitrophenyl phosphate reverses rapidly. The inhibitor ratio will be influenced by the relative rates of reversal of true and pseudo cholinesterase.

hydrolysed by the enzyme. If the mechanism of hydrolysis of acetylcholine by cholinesterase put forward by Bergmann, Wilson & Nachmansohn (1950) is accepted and the ester is attached to the ester-attracting site by the electrophilic carbon of the acyl group, then it is highly probable that the organo-phosphorus compounds are attached by the electrophilic phosphorus (Nachmansohn & Wilson, 1951). If this is so, the length of the alkoxyl chains attached to the phosphorus should play an important part in the 'fit' of these compounds on the enzyme surface in a similar way to the effect of various acyl groups on the rate of hydrolysis of choline esters. In Table 5 the substrate ratios for various choline esters are compared with the inhibitor ratio for inhibitors with similar lengths of groups from the electrophilic group. It should be pointed out that the substrate ratio is the rate of hydrolysis (based on acetylcholine = 100) for pseudo over that for true cholinesterase while the inhibitor ratio is the concentration of inhibitor for 50 % inhibition for true cholinesterase over that for pseudo cholinesterase. The ratios have been calculated in this way because if the inhibitor is a substrate for the enzyme then the more efficiently it is hydrolysed by the enzyme the lower will be the concentration of inhibitor necessary for inhibition. It appears from a limited number of inhibitors that there is a rough correlation. For instance, on passing from *n*-butyrylcholine to *iso*valerylcholine the substrate ratio changes from 94 to 263. A change of the inhibitor ratio in the same direction is shown for three groups of inhibitors: tetraethyl and tetra-*iso*propyl pyrophosphate, diethyl and di*iso*propyl *p*-nitrophenyl phosphates and diethyl and di*iso*-propyl fluorophosphonates. There is a marked change in substrate ratio from *iso*butyrylcholine to *iso*valerylcholine. A similar change in inhibitor ratio is found from bisdimethylamino to bismono-*iso*propylamino fluorophosphine oxides.

DISCUSSION

Evidence has been presented that in the differential inhibition of true and pseudo cholinesterase by a series of organo-phosphorus compounds an important factor is the alkyl group attached to the phosphorus. Changes in the acyl group of choline esters produce marked changes in the relative rates of hydrolysis of these esters by true and pseudo cholinesterases. Organo-phosphorus inhibitors have been compared with choline esters with regard to length of the alkoxyl and acyl chains attached to the electrophilic phosphorus and carbon respectively. Considering a choline ester and an inhibitor with groups of a similar length, if the choline ester after a change of acyl group is hydrolysed relatively more readily by pseudo than true cholinesterase, then with a similar change of groups (similar as regards length) on the inhibitor a compound is produced which is relatively more efficient against pseudo than true cholinesterase. It has previously been shown, by using a series of inhibitors of widely varying stabilities to hydrolysis but with the alkyl groups maintained constant (Aldridge & Davison, 1952*a*, *b*), that the more stable an inhibitor is to hydrolysis the less effective it is as an inhibitor. It appears that at least two factors are involved in the efficiency of an organo-phosphorus compound as an inhibitor; its stability to hydrolysis and the particular groups attached to the phosphorus.

These observations are consistent with the view that organo-phosphorus inhibitors are substrates for cholinesterase, are hydrolysed and the phosphorus remains attached to the enzyme. Changes in the alkyl groups of an inhibitor should alter the 'fit' of the enzyme surface in a similar way that changes in the acyl groups of choline esters affect their hydrolysis by the enzyme. On the Fischer 'lock and key' principle the better the 'fit' of a substrate on the enzyme surface the more readily it is hydrolysed. In a similar way the better the 'fit' of an organo-phosphorus inhibitor the more readily it should be hydrolysed and the more efficient it should be as an inhibitor. Stability to hydrolysis of the inhibitor is also an important factor which should alter the ease with which it is hydrolysed at the enzyme surface. Such a view of the mechanism of inhibition places most of the responsibility for inhibition on the enzyme itself. This would be consistent with the fact that some stable organo-phosphorus compounds do inhibit cholinesterase. Diethyl phenyl phosphate with a half life of 8 years in phosphate buffer, pH 7·6, and at 37° does inhibit cholinesterase at 10^{-3} M concentrations (Aldridge & Davison, 1952*a*,*b*). This mechanism of inhibition is similar to that elucidated by Jansen *et al.* (1949, 1950) for the inhibition of chymotrypsin by DFP.

Although isopestox and ISO-OMPA are good inhibitors for the *in vitro* differentiation of true and pseudo cholinesterase in some species, Table 3 shows that caution must be used in the use of inhibitors for this purpose. Substrates have also been used to differentiate true and pseudo cholinesterase, but evidence is accumulating that this method is far from certain (Ellis, 1947; Metcalf & March, 1950; Levine & Suran, 1950; Earl & Thompson, 1952). It has been suggested that enzymes of the same type from different species will show a different spectrum of activities against different substrates (Augustinsson, 1951; Aldridge, 1953*b*). If this is accepted, the view that organo-phosphorus inhibitors are substrates for cholinesterase would be consistent with the finding that variations in the response of true and pseudo cholinesterases of different species to these inhibitors have been obtained.

SUMMARY

1. The inhibition of true and pseudo cholinesterase by a series of organo-phosphorus inhibitors has been determined.

2. Based on the effect of changes of acyl groups in choline esters on their hydrolysis by true and pseudo cholinesterase an explanation of the different sensitivities of the two enzymes for organo-phosphorus inhibitors has been suggested.

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Studies on Fumarase

2. THE EFFECTS OF INORGANIC ANIONS ON FUMARASE ACTIVITY

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It was shown quite early in the history of this enzyme that the activity of fumarase is affected by the addition of inorganic salts. Mann & Woolf (1930), working with whole washed cells of *Escherichia coli*, found that sulphates inhibited slightly the hydration of fumaric acid to L-malic acid; while phosphates and citrates strongly activated this reaction. Similarly, the activating effect of phosphates on the extracted enzyme from animal tissues has been shown by various workers (Quastel, 1931; Clutterbuck, 1928; Davydova, 1947). In this paper is reported the effect of various anions on the activity of crystalline fumarase.

METHODS

The enzyme used was obtained from pigs' hearts by the method already described (Massey, 1951, 1952) and was recrystallized twice before use. The enzyme activity was determined by the spectrophotometric method of Racker (1950), which depends on the formation or disappearance of the double bond of fumaric acid. The fumarate or L-malate concentrations used in these experiments were generally 0·0167 M-fumarate or 0·0835 M-L-malate. These concentrations are over five times the highest Michaelis constant observed with either substrate. The rate of the reaction is linear at first and then falls off as the substrate concentration decreases and the product is formed (Fig. 1). The values

reported here are the initial velocities, when the reaction rate is linear, and may be expressed as change in fumarate concentration per unit time or as change in optical density per unit time. As there is a direct correlation between the fumarate concentration and the optical density (Massey, 1953), most of the values reported here are, for the sake of convenience, expressed as changes in optical density. Unless otherwise stated, all the results described here were obtained at room temperature.

RESULTS

The effect of pH *on the activity of well dialysed fumarase.* Contrary to previous claims (Laki, 1941), fumarase when pure is not denatured by exhaustive dialysis against glass-distilled water. When impure,

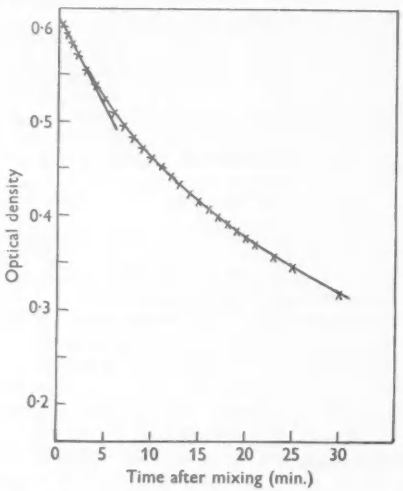

Fig. 1. The course of hydration of fumarate to L-malate by a small amount of fumarase ($\lambda = 2950$ A.).

however, it does precipitate out in an inactive form on dialysis, possibly owing to absorption on a contaminating protein which is insoluble in distilled water. The pH-activity curves of well dialysed solutions of pure enzyme, in the presence of L-malic or fumaric acids neutralized to the desired pH, are shown in Fig. 2. Below pH 7, fumarate behaves as a reasonably good buffer; L-malate is an efficient buffer up to about pH 8, so that the enzyme activity can be determined at constant pH without the addition of any anions other than the substrates. The pH values of the reaction mixtures were checked before the addition of enzyme and during the course of the reaction. Under the conditions used, with small concentrations of enzyme the pH never varied more than 0·1 pH unit during the period of reaction, and in most cases much less than this.

Fumarase in the absence of inorganic anions has quite different pH-activity curves with its two substrates (Fig. 2). With fumarate as substrate, the curve is rather flat with a poorly defined optimum around pH 5·6. However, with L-malate, a symmetrical pH curve with a well defined optimum at pH 7·2 is obtained.

The effect of phosphate. Fig. 2 also shows the effect of 0·06 M-phosphate on the pH-activity curves of fumarase with both fumarate and L-malate as substrates. With both substrates the pH optimum is shifted toward the alkaline side, and there is considerable activation over most of the pH range studied.

The effects of phosphate on the hydration reaction are very similar to those found with whole bacteria by Mann & Woolf (1930). The shape of the pH curve in the presence of phosphate, and the pH optimum, are the same as they obtained. The pH

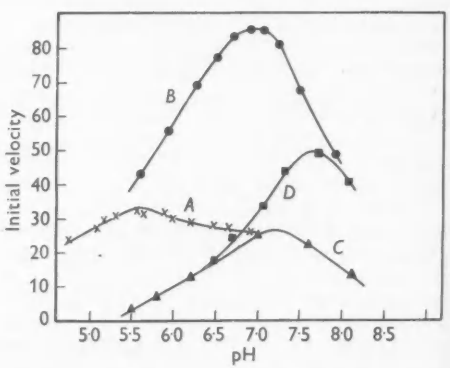

Fig. 2. The effect of pH on fumarase activity in the presence and absence of phosphate. *A*, 0·0167 M-fumarate in the absence of phosphate; *B*, 0·0167 M-fumarate in the presence of 0·06 M-phosphate; *C*, 0·0835 M-L-malate in the absence of phosphate; *D*, 0·0835 M-L-malate in the presence of 0·06 M-phosphate.

optimum (5·6) of crystallized fumarase in the absence of inorganic salts is considerably lower than the value (6·4) obtained by Mann & Woolf (1930) with washed bacteria. This difference may well be due to the retention of some phosphate by the bacteria even after thorough washing.

Table 1 shows the effect of phosphate concentration on the extent of activation, using 0·0835 M-L-malate as substrate at pH 7·4. Maximum activation is quickly reached and is maintained over a considerable range of phosphate concentration. At high phosphate concentrations, however, the activity again falls off. Such effects were found at all pH values investigated.

That the activating effects of phosphate are not due to changes in the affinity of the enzyme for the substrate are shown in Fig. 3 for fumarate as substrate at pH 6·35, and 23°. The initial velocities over a range of fumarate concentration from M/600 to M/120 are plotted according to the method of Lineweaver & Burk (1934). It is evident that under these conditions, while the enzymic activity is greatly increased by phosphate, the Michaelis constant is unaffected.

Table 1. *The effect of concentration of phosphate on activity of fumarase at* pH 7·4

(The activity values represent the initial increase in optical density over a period of 3 min. when 0·0835 M-L-malate is acted on by a constant quantity of salt-free fumarase.)

Concentration of phosphate (M)	Activity
0	37
0·00033	44
0·00066	47
0·00133	49
0·0020	50
0·0033	53
0·0066	55
0·0167	56
0·0835	56
0·167	51
0·333	40

Again, the pH-activity curves in the presence of phosphate are not greatly influenced by the ionic strength. The results shown in Fig. 2 are with 0·06 M-phosphate solutions in which the ionic strength varies from 0·06 to 0·18 over the range of pH studied. If, however, the ionic strength of the phosphate is maintained constant at 0·06, the pH-activity curve remains unaltered.

The effect of other anions. Several other anions affect the activity of well dialysed solutions of fumarase. The effects of chloride, sulphate, selenate, arsenite, citrate, borate, phosphate and arsenate are shown in Fig. 4. By comparison it should be noted that with washed bacteria Mann & Woolf (1930) found sulphate slightly inhibitory, and citrate an activator even more efficient than phosphate. These differences may be due to different properties of bacterial and animal fumarases, or more probably are connected with permeability factors. Divalent ions activate to a lesser extent than trivalent ions, and the greater the extent of activation, the further toward the alkaline the pH optimum is shifted. Chloride ions, which act as inhibitor over most of the pH curve, while also shifting the pH optimum considerably toward the alkaline, appear to have little effect on the alkaline branch of the pH-activity curve, but have a very large effect on the acidic branch. Bromide, iodide and thiocyanate ions behave in the same way as chloride. The inhibition with these monovalent ions occurs also in the presence of phosphate; Fig. 5 shows their effect

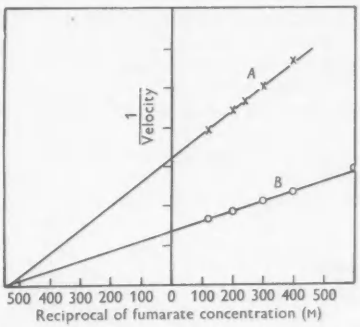

Fig. 3. Determination of the Michaelis constant of fumarase with fumarate as substrate at pH 6·35 and 23°. *A*, in the absence of phosphate; *B*, in the presence of 0·06 M-phosphate.

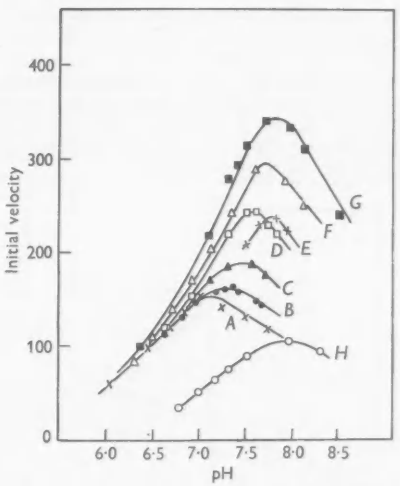

Fig. 4. The effect of some anions on the dehydration of L-malate by fumarase. Concentration of L-malate, 0·0835 M. *A*, in the absence of added anions; *B*, 0·03 M-sodium selenate or 0·025 M-sodium sulphate; *C*, 0·01 M-sodium arsenite; *D*, 0·01 M-sodium citrate; *E*, 0·2 M-sodium borate; *F*, 0·06 M-sodium and potassium phosphate; *G*, 0·05 M-sodium arsenate; *H*, 0·1 M-sodium chloride.

under similar conditions, but in the presence of 0·06 M-phosphate. That the inhibition, like the activation with phosphate, has no effect on the Michaelis constant is shown in Fig. 6.

That the activation produced by phosphate, arsenate, etc., may be due to the removal of a contaminating inhibitory metal is unlikely for several reasons. First, all the reactions reported here have been obtained in solutions employing glass-distilled water in which the concentration of heavy metals is extremely minute. Secondly, 0·033 M-versene (ethylenediaminetetraacetic acid), which is an extremely strong metal-chelating agent, has no effect on the salt-free reaction at pH's 6·3 or 7·9. Similarly, diethyldithiocarbamate in saturated aqueous solution at these pH values has no effect.

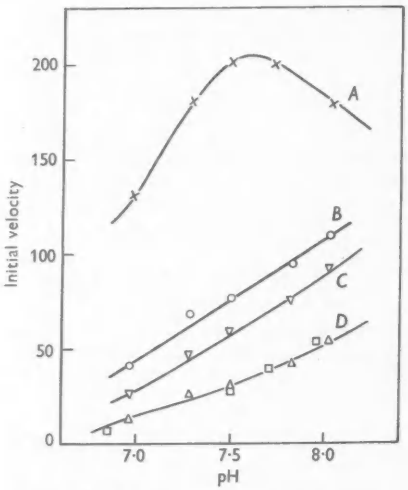

Fig. 5. The effect of some monovalent anions on fumarase activity. *A*, L-malate (0·0835 M) in the presence of 0·06 M-phosphate; *B*, plus 0·1 M-potassium chloride; *C*, plus 0·1 M-potassium bromide; *D*, plus 0·1 M-potassium iodide △ or 0·1 M-ammonium thiocyanate, □.

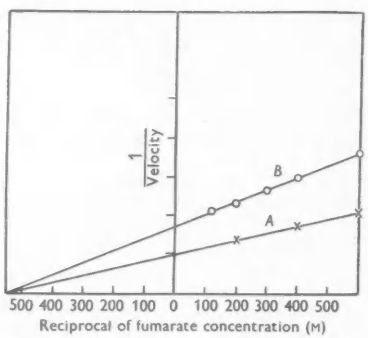

Fig. 6. Determination of the Michaelis constant of fumarase with fumarate as substrate at pH 6·35, and 23°. *A*, in the presence of 0·06 M-phosphate; *B*, in the presence of 0·06 M-phosphate plus 0·01 M-ammonium thiocyanate.

DISCUSSION

The results shown here might be explained in terms of the theory of Michaelis & Davidsohn (1911) and Michaelis & Pechstein (1914) that the dissociation of two particular groups, acidic and basic, determines the activity of an enzyme, and that the pH-activity curve is determined by the dissociation constants of these groups.

According to this theory, the optimum activity is at that pH value where the dissociation of the two groups responsible for activity is maximal. It is obvious that the experimental results which support this theory do not really enable us to argue that only one acidic and one basic group are concerned in the activation; theoretically, any number of groups which had pK values close to each other would produce the same results.

Applied to fumarase, it is seen that, with both fumarate and L-malate, it is the alkaline branch of the pH-activity curves which is predominantly altered by the addition of phosphate. This suggests that the basic dissociation constant(s) of the enzyme is affected by the phosphate, rather than the acidic.

Fig. 2 also shows that, in the absence or in the presence of phosphate, the alkaline branches of the activity curves for both fumarate and L-malate are close together, while the acid branches are widely separated. This suggests that the two substrates may also affect the dissociation constants responsible for activation, except that now the effect is on the acidic dissociation constant(s) rather than on the basic. There is evidence (Massey, unpublished) that the substrates and other carboxylic acids are capable of secondary combinations with fumarase at sites other than the active centre(s), and that this type of combination affects the dissociation constants of groups at the active centre.

Effects similar to those of phosphate are shown with all the activating ions investigated. All of the anions found to activate thus appear to alter the basic dissociation constant(s), while having little effect on the acidic dissociation constant(s).

With the inhibiting monovalent anions, the effect seems to be of the opposite kind; here the acidic dissociation constant(s) seem to be affected much more than the basic dissociation constant(s).

It is rather difficult to visualize, however, how the interaction of protein and anion can lead to a shifting of the basic dissociation constant toward the alkaline region, as occurs with the activating ions. Binding of an anion should lead to the suppression of the ionization of a basic group, with a consequent

shift of the basic dissociation constant toward acid pH values. It is also difficult to explain why monovalent ions such as chloride seem to have no effect on the basic dissociation constant, but do affect the acidic dissociation constant; the shift again being toward the alkaline.

Presumably the effects described cannot be due to a direct influence of the anions on the active centre. This has been shown directly for phosphate and thiocyanate at pH 6·35; at this pH neither anion has any effect on the Michaelis constant.

Such shifts may be due to combination of the anions with basic groups adjacent to the acidic and basic groups responsible for activation. The combination of an anion with a basic group adjacent to the activating basic group might be expected to cause an increased ionization of the activating basic group, i.e. the dissociation constant of this group would be shifted toward the alkaline and activation would result over the alkaline range of the pH-activity curve.

If, on the other hand, the anion combined with a basic group adjacent to the acidic group causing activation, then the dissociation of this acidic group would be made more difficult, and the dissociation constant would be shifted toward the alkaline, with a consequent inhibition, especially over the acid branch of the pH-activity curve.

However, while this interpretation fits the experimental data, it gives little clue as to why some anions should combine preferentially with basic groups adjacent to the activating acidic group, and why others combine preferentially with basic groups adjacent to the activating basic group.

This preferential adsorption at certain sites in the enzyme may be due to differences in size or charge of the anions; all the inhibiting anions are rather small and are monovalent, while the activating anions are much larger and are at least divalent.

However, some experiments with fumarase have shown that some of the changes in activity obtained on the addition of anions can be correlated with gross changes of molecular arrangement of the enzyme. Thus, changes induced in the dissociation constants of the protein, if they do occur, are probably not the only factors effecting the changes in activity.

SUMMARY

1. The effect of pH on fumarase activity in the absence of inorganic anions is described.

2. Sulphate (and selenate), arsenite, citrate, borate, phosphate and arsenate, in increasing order of effectiveness, activate salt-free fumarase and alter the pH of optimal activity.

3. Chloride, bromide, thiocyanate and iodide, in increasing order of effectiveness, inhibit the action of fumarase, either in the presence or absence of activating anions. The extent of the inhibition is markedly dependent on the pH.

I am indebted to Dr M. Dixon, F.R.S., for his advice and encouragement, and to Dr G. Weber and Mr D. Lawrence for many helpful discussions. The work was carried out with a grant from the Commonwealth Scientific and Industrial Research Organization, Australia.

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Studies on Fumarase

3. THE EFFECT OF TEMPERATURE

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A study of the variation with temperature and pH of such properties of an enzyme as its maximum velocity and affinities for substrates and inhibitors might be expected to throw valuable light on the mechanism of the reaction catalysed by the enzyme. In this paper are reported some results on the variation of maximum velocity and Michaelis constants with temperature of crystalline fumarase, as well as the variation of Michaelis constants with pH. The variation of activity with pH in the presence of various salts has already been reported (Massey, 1952), and the variation of inhibitory constants of a number of dicarboxylic acids with both temperature and pH will be reported in a future paper.

METHODS

Fumarase. The fumarase solutions used were of twice recrystallized material obtained from pigs' hearts as already described (Massey, 1952).

Estimation of enzyme activity. Details are given in the second paper of this series (Massey, 1953). All the values reported here are initial velocities, when the reaction rate is still constant.

Activating anions. All the experiments reported here, unless stated to the contrary, were carried out in the presence of 0·067 M-phosphate buffers.

Temperature control. The temperature was controlled to within $\pm 0{\cdot}1°$ of the desired temperature by means of an apparatus designed by Dr M. Dixon, F.R.S., which will be described in a separate communication.

Determination of the ionization constants of fumaric acid. These were determined by electrometric titration of 0·02 M-fumaric acid with CO_2-free 0·025 N-NaOH. The temperature was maintained constant to within $\pm 0{\cdot}1°$ by immersion in a thermostated water bath. Stirring of the reaction mixture was accomplished by a constant stream of CO_2-free N_2, to prevent the absorption of CO_2 from the atmosphere into the reaction mixture. The titration was followed by measuring the pH with a 'Cambridge' electrometer, the power for which was supplied by a 12 V. storage battery. The instrument was standardized at the particular temperature investigated with 0·05 M-potassium hydrogen phthalate buffer, the pH value of which was taken to be 4·00 at all the temperatures investigated. The instrument was checked for drift at the end of the titration; only in those cases where the drift was less than $\pm 0{\cdot}05$ pH unit were pK values calculated from the results.

The pK values reported here were calculated from the pH values of the mid points of the integral sections of the titration curve (see Britton, 1942).

Determination of Michaelis constants. Michaelis constants (K_m) were determined by the method of Lineweaver & Burk (1934).

RESULTS

The temperature coefficient of fumarate absorption. The absorption of fumarate in the ultraviolet is quite markedly temperature dependent. Fig. 1

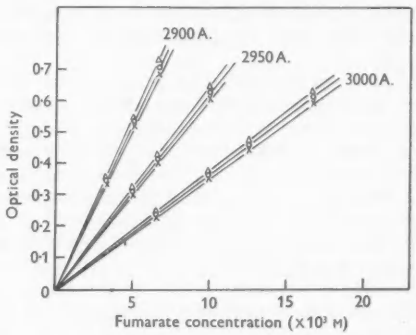

Fig. 1. The variation of optical density of sodium fumarate at pH 7·0 with temperature. Δ, 40°; ○, 30°; ×, 20°. Thickness of cell 1 cm.

shows the effect of temperature on the absorption of fumarate at several wavelengths. The proportionate increase in absorption with temperature at these wavelengths seems to be independent of the fumarate concentration; it amounts to a 3·5 % increase in optical density, per 10° rise in temperature. The absorption of fumarate seems to be independent of pH over the range pH 5·5 to pH 9; below pH 5, however, the absorption increases, this being due probably to the partially ionized form of the acid.

The extent of variation of the absorption of fumarate with temperature is insufficient to warrant correction values being applied to all results obtained. Accordingly, the experimental points in the graphs shown here have not been corrected for change in absorption with temperature. However, a correction factor has been applied to all calculations based on these graphs.

The effect of pH *on the Michaelis constants.* Fig. 2 shows the effect on the Michaelis constants, K_m, of

altering the pH. The K_m for fumarate is much more pH-sensitive than that for L-malate, but for both a definite maximum is reached at a particular pH. These maxima, for both substrates, occur at approximately the same pH values as the optimal activities under the same conditions. Thus the pH optimum of fumarase in the presence of 0·067 M-phosphate with fumarate as substrate is pH 6·95 (Massey, 1953); the pH of maximum K_m is about 7·0. Similarly, the optimal activity under the same conditions, but with L-malate as substrate, is about pH 7·7, and from Fig. 2, the pH of maximum K_m for L-malate is around 8·0.

Similar slopes are obtained if the fumarate concentration is doubled. The effect of temperature when fumarate is substrate is of three different types. At high pH values results of the type shown in Fig. 4 are obtained, in which the activation energy at first remains constant as the temperature is increased, to change sharply at a particular temperature to a higher activation energy. At pH values around the optimum no change occurs in activation energy over the whole range of temperature investigated (Fig. 5). At low pH values (Fig. 6), the converse of what happens at high pH values occurs. Here, however, the temperature of changeover from one

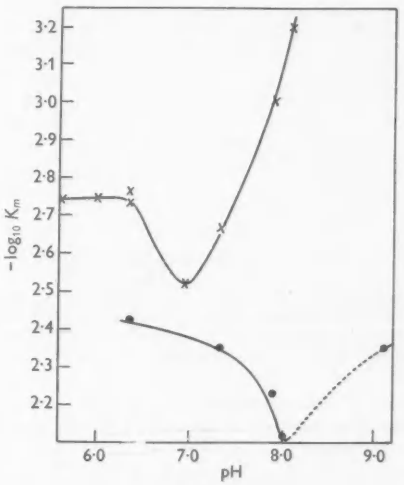

Fig. 2. The variation of Michaelis constants of fumarase with pH at 23°. —×—×—, fumarate as substrate; —●—●—, malate as substrate.

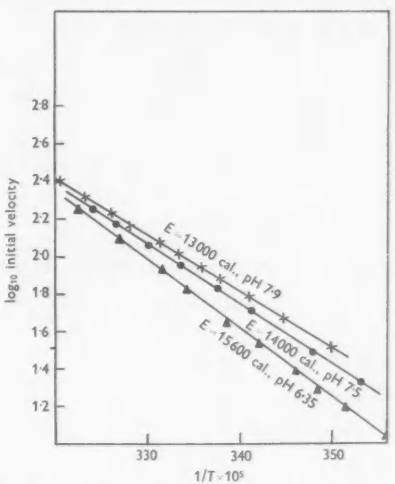

Fig. 3. The variation of the initial velocity of dehydration reaction of fumarase with temperature. Sodium L-malate concentration, 0·033 M.

The effect of temperature on reaction velocity. Fig. 3 shows the effect of temperature on the initial rate of conversion of L-malate to fumarate at various pH values when the initial concentration of L-malate was 0·033 M. The slopes shown are with different quantities of enzyme for the different pH values used in order to keep the velocities at convenient levels. These results show a high temperature coefficient indicating a high energy of activation as obtained from the Arrhenius equation. There is very little change in the energy of activation over the pH range studied, varying from 13100 cal. at pH 7·94, through 14000 cal. at pH 7·52 to 15600 cal. at pH 6·35.

Figs. 4–6 show the effect of temperature in different pH ranges when the substrate is 0·0167 M-fumarate. As with the values for L-malate, these results are with different quantities of enzyme.

activation energy to the other seems to be independent of pH, occurring in the range 17–19°. Changes of the type shown in Fig. 6 have been found in the last few years with several enzymes (see reviews by Stearn, 1949; Sizer, 1943), but no temperature effects of the type shown in Fig. 4 have been found previously with any enzyme.

The values for the activation energies calculated from these graphs are shown in Table 1, and on the graphs themselves.

The changeover from a lower to a higher activation energy with increase in temperature is also shown with L-malate as substrate at very high pH values. Fig. 7 shows the effect of temperature on the dehydration of L-malate at pH 9·0 in the presence of 0·187 M-borate. Here the critical temperature is at 32° and the activation energies below and above this temperature are 9300 and 14800 cal.

However, no indication has ever been obtained of L-malate behaving in the same way as fumarate at lower pH values, where the opposite effect occurs.

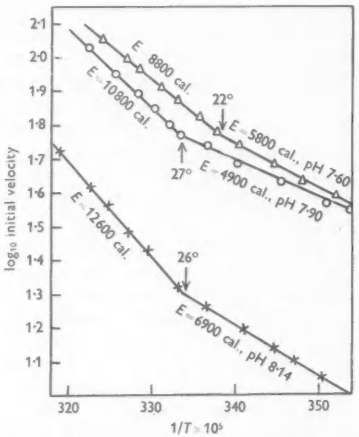

Fig. 4. The variation with temperature of the initial velocity of the hydration reaction of fumarase at alkaline pH values. Sodium fumarate concentration, 0·0167 M.

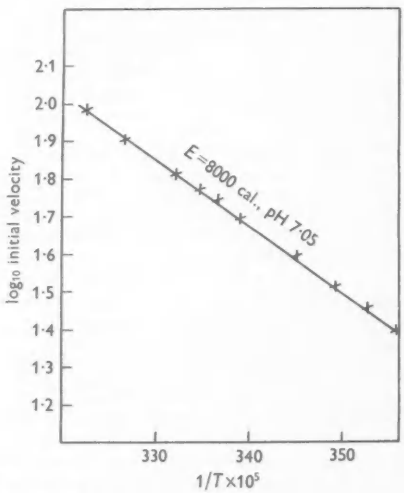

Fig. 5. The variation with temperature of the initial velocity of the hydration reaction of fumarase at pH 7. Sodium fumarate concentration, 0·0167 M.

The fact that the behaviour with fumarate and L-malate is so different at low pH values suggests that under these conditions the concentration of water in a form capable of taking part in the reaction may be limiting over a certain temperature range. For example, it is possible that heavily hydrogen-bonded water may be incapable of orientation on the enzyme protein molecule in the way necessary for the hydration reaction to proceed. The breakage of hydrogen bonding in liquid

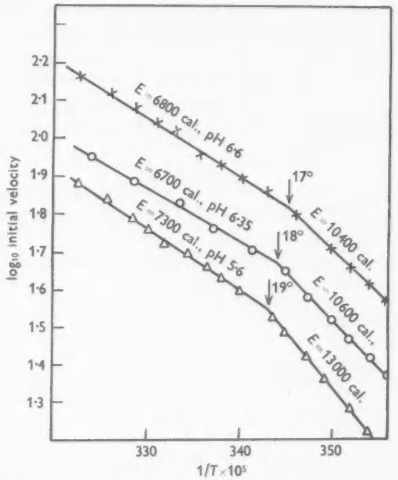

Fig. 6. The variation with temperature of the initial velocity of the hydration reaction of fumarase at acid pH values. Sodium fumarate concentration, 0·0167 M.

Table 1. *The variation of activation energy of fumarase with temperature and* pH *with fumarate as substrate*

pH	Activation energy at lower temp. (cal.)	Activation energy at higher temp. (cal.)	Critical temp. (°)
8·14	6 900	12 600	26
7·9	4 900	10 800	27
7·6	5 800	8 800	22
7·05	8 000	8 000	None
6·6	10 400	6 800	17
6·35	10 600	6 700	18
5·6	13 000	7 300	19
5·3	13 700	—	—

water increases rapidly with temperature; it is possible that at low temperatures sufficient hydrogen bonding exists to prevent the reaction proceeding at the maximum rate. If such a premise were true, the addition of substances capable of breaking the hydrogen bonds between water molecules, should abolish the change from one activation energy to another, or should at least lower the temperature at which such a break normally occurs. Methanol and ethanol are known to be capable of such an action. Fig. 8 shows the effect of 10% methanol on the temperature coefficients with

fumarate as substrate. The effect of methanol is to abolish the changes in activation energy already mentioned, and at the same time to increase the

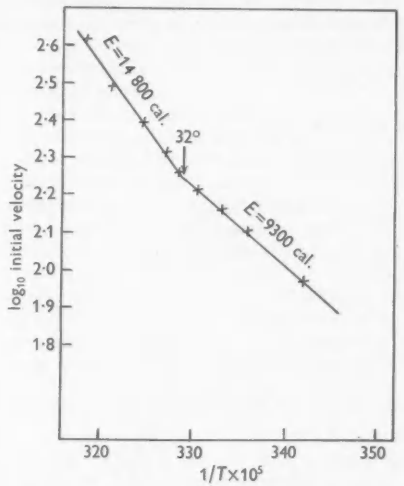

Fig. 7. The variation with temperature of the initial velocity of the dehydration reaction of fumarase in the presence of 0·187 M-sodium borate, pH 9·0. Concentration of sodium L-malate, 0·033 M.

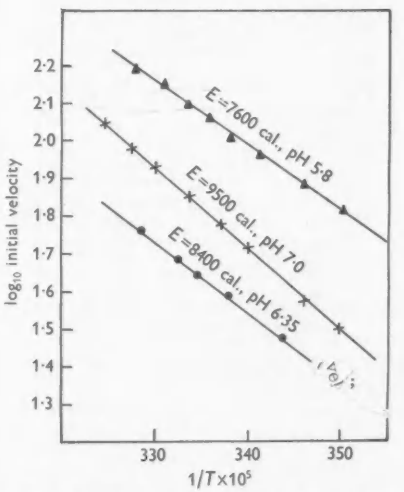

Fig. 8. The variation with temperature of the initial velocity of the hydration reaction of fumarase in the presence of 10% methanol. Sodium fumarate concentration, 0·0167 M.

apparent energy of activation. The results with methanol and ethanol are complicated by the fact that their presence causes the phosphate buffers to assume quite an appreciable temperature coefficient. Their effect is to decrease the pH of the buffer as the temperature is raised. As most of the results shown in Fig. 8 are at pH values below the optimum, the activation energies calculated from the slopes in Fig. 8 will in fact be smaller than the real values. Methanol (10%) has a similar effect when L-malate is the substrate. For example, at pH 7·6 in the absence of methanol the activation energy is 13 600 cal., in the presence of 10% methanol it is 14 800 cal.; a correction made for change in pH with temperature when methanol is present would bring this value to about 16 000 cal.

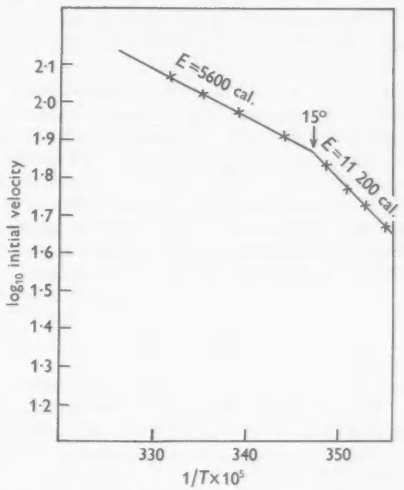

Fig. 9. The variation with temperature of the initial velocity of the hydration reaction of fumarase in the presence of 10% ethanol at pH 6·5. Sodium fumarate concentration, 0·0167 M.

The effect of methanol cannot be due to breaking of hydrogen bonds between molecules of water, as ethanol which also reduces the hydrogen bonding has no effect in producing a single activation energy over the range of temperature studied, although its presence does appear to lower the critical temperature at which the break occurs (Fig. 9).

The ionization constants of fumaric acid. It was considered possible that the explanation for the change of activation energy at low pH values when fumarate is substrate might be in some anomalous property of fumaric acid, such as a sharp change in the ionization constant at 18°. If fumarase were to combine preferentially with one of the ionic forms of fumaric acid such a change would go a long way toward providing an explanation for this phenomenon. Accordingly, the ionization constants of twice recrystallized fumaric acid were determined

However, no indication has ever been obtained of L-malate behaving in the same way as fumarate at lower pH values, where the opposite effect occurs.

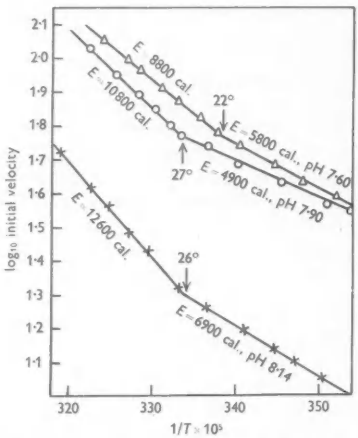

Fig. 4. The variation with temperature of the initial velocity of the hydration reaction of fumarase at alkaline pH values. Sodium fumarate concentration, 0·0167 M.

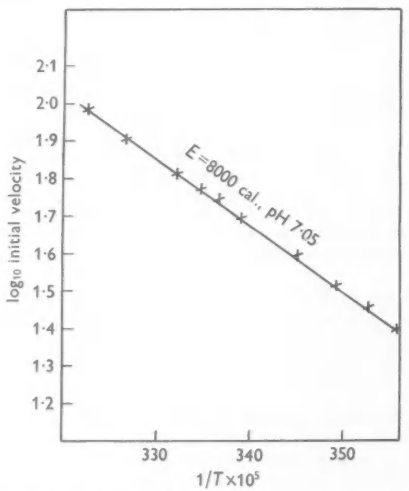

Fig. 5. The variation with temperature of the initial velocity of the hydration reaction of fumarase at pH 7. Sodium fumarate concentration, 0·0167 M.

The fact that the behaviour with fumarate and L-malate is so different at low pH values suggests that under these conditions the concentration of water in a form capable of taking part in the reaction may be limiting over a certain temperature range. For example, it is possible that heavily hydrogen-bonded water may be incapable of orientation on the enzyme protein molecule in the way necessary for the hydration reaction to proceed. The breakage of hydrogen bonding in liquid

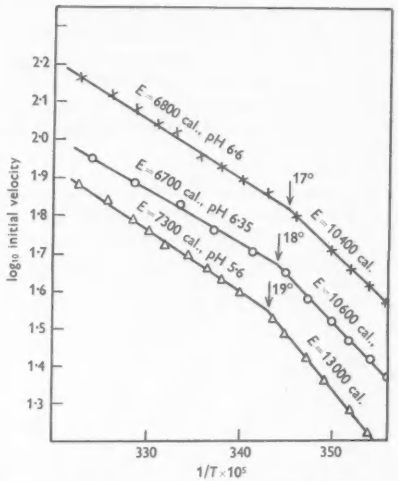

Fig. 6. The variation with temperature of the initial velocity of the hydration reaction of fumarase at acid pH values. Sodium fumarate concentration, 0·0167 M.

Table 1. *The variation of activation energy of fumarase with temperature and* pH *with fumarate as substrate*

pH	Activation energy at lower temp. (cal.)	Activation energy at higher temp. (cal.)	Critical temp. (°)
8·14	6 900	12 600	26
7·9	4 900	10 800	27
7·6	5 800	8 800	22
7·05	8 000	8 000	None
6·6	10 400	6 800	17
6·35	10 600	6 700	18
5·6	13 000	7 300	19
5·3	13 700	—	—

water increases rapidly with temperature; it is possible that at low temperatures sufficient hydrogen bonding exists to prevent the reaction proceeding at the maximum rate. If such a premise were true, the addition of substances capable of breaking the hydrogen bonds between water molecules, should abolish the change from one activation energy to another, or should at least lower the temperature at which such a break normally occurs. Methanol and ethanol are known to be capable of such an action. Fig. 8 shows the effect of 10% methanol on the temperature coefficients with

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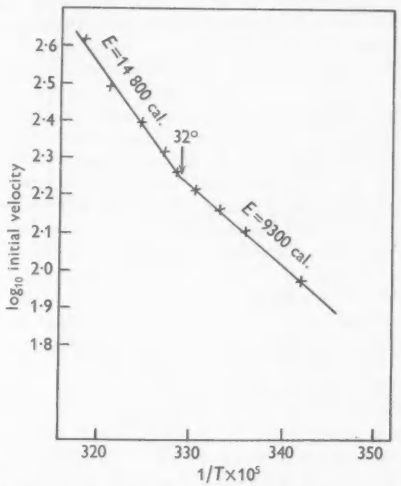

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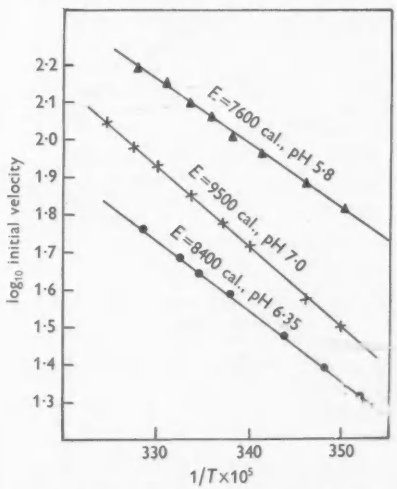

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apparent energy of activation. The results with methanol and ethanol are complicated by the fact that their presence causes the phosphate buffers to assume quite an appreciable temperature coefficient. Their effect is to decrease the pH of the buffer as the temperature is raised. As most of the results shown in Fig. 8 are at pH values below the optimum, the activation energies calculated from the slopes in Fig. 8 will in fact be smaller than the real values. Methanol (10%) has a similar effect when L-malate is the substrate. For example, at pH 7·6 in the absence of methanol the activation energy is 13 600 cal., in the presence of 10% methanol it is 14 800 cal.; a correction made for change in pH with temperature when methanol is present would bring this value to about 16 000 cal.

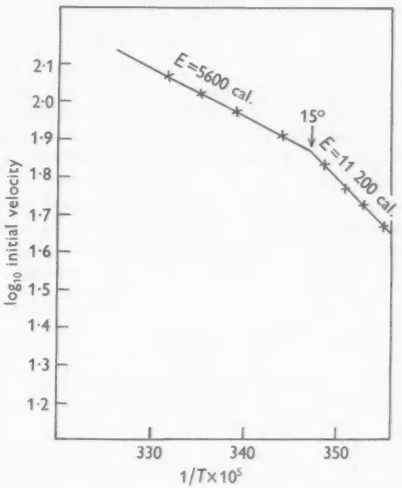

Fig. 9. The variation with temperature of the initial velocity of the hydration reaction of fumarase in the presence of 10% ethanol at pH 6·5. Sodium fumarate concentration, 0·0167 M.

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The ionization constants of fumaric acid. It was considered possible that the explanation for the change of activation energy at low pH values when fumarate is substrate might be in some anomalous property of fumaric acid, such as a sharp change in the ionization constant at 18°. If fumarase were to combine preferentially with one of the ionic forms of fumaric acid such a change would go a long way toward providing an explanation for this phenomenon. Accordingly, the ionization constants of twice recrystallized fumaric acid were determined

at various temperatures by electrometric titration in an atmosphere of nitrogen. The results are shown in Table 2. There is no evidence from these results to suggest that the changes in activation energy with temperature are linked with the ionization constants of fumaric acid.

Table 2. *The effect of temperature on the ionization constants of fumaric acid*

Temperature (°)	pK_1	pK_2
4	3·02	4·24
	3·04	4·26
17·5	3·05	4·31
32	3·00	4·30
35	3·03	4·35

The effect of temperature on Michaelis constants. At a given temperature the Michaelis constants of fumarase vary with pH in the same way as the initial velocities. At a given pH the K_m will also vary with temperature. It should be possible, by maintaining the pH constant, and studying the variation with temperature of maximum initial velocities, Michaelis constants and equilibrium constants, to obtain a thermodynamic picture of the enzymic reaction.

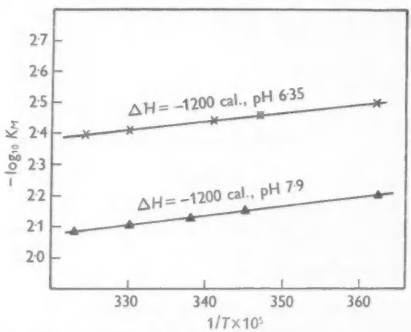

Fig. 10. The variation with temperature of the Michaelis constant of fumarase and L-malate.

Fig. 10 shows the effect of temperature on the K_m with L-malate as substrate at pH's 6·35 and 7·9. Here the K_m increases as the temperature is increased, indicating that heat is evolved in the formation of the enzyme-malate complex. The ΔH value calculated from Fig. 11 is -1200 cal./mole at both pH values.

Fig. 11 shows the effect of temperature on the K_m with fumarate as substrate at pH's 6·5, 6·35 and 7·9. Here, as for the initial velocities with fumarate as substrate, at low pH values there is a critical temperature with different heats of formation of the enzyme-substrate complex above and below this temperature. At pH 5·6 there is a break at 18° with a ΔH value below this temperature of 800 cal./mole and a ΔH value of 7100 cal./mole above this temperature. At pH 6·35 the break is again at 18° and the ΔH values above and below this temperature are 4200 and 400 cal./mole respectively. At pH 8·0 the heat of formation of the Michaelis complex is 4200 cal./mole over the whole of the temperature range investigated (3–35°). It is interesting to note that whereas the formation of the fumarase-malate complex proceeds with evolution of heat, fumarase-fumarate complexes absorb heat in their formation.

It is immediately striking that at low pH values in the presence of fumarate, the critical temperature is the same both for initial velocities and for Michaelis constants.

Also at pH's 5·6 and 6·35, the differences between the ΔH values above and below the critical temperature are very similar to the difference in energy of activation (E) calculated from the changes of initial velocity with temperature. Thus at pH 5·6 the difference in ΔH about the critical temperature is 6300 cal./mole while the change in E about this temperature is 5700 cal. (see Fig. 6). Similarly, at pH 6·35 the difference in ΔH is 3800 cal./mole while the difference in E is 3900 cal. At pH 8·0 there is no

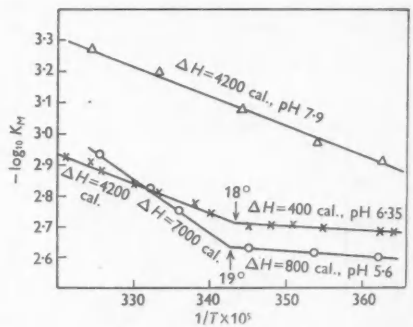

Fig. 11. The variation with temperature of the Michaelis constant of fumarase and fumarate.

change of K_m corresponding to that found with initial velocity.

The effect of temperature on equilibrium. Fig. 12 shows the variation with temperature of the equilibrium constant $\left(K = \frac{[\text{malate}]}{[\text{fumarate}]}\right)$ at pH's 6·35 and 7·3. The equilibrium is unaltered by pH over the range studied, and the ΔH of the overall reaction is calculated from the graph to be 3600 cal./mole. This is in good agreement with the values reported previously (Scott & Powell, 1948; Ohlmeyer, 1945).

The heat changes of the overall reaction. The data reported give a very suggestive picture of the heat

changes of the enzyme reaction which may throw some light on the mechanism of the reaction. The changes of initial velocity with temperature represent changes in maximum initial velocity, as the substrate concentrations used were never less

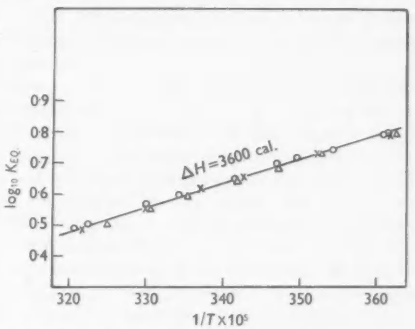

Fig. 12. The effect of temperature on the equilibrium constant $K=\frac{[\text{malate}]}{[\text{fumarate}]}$. ×, starting with sodium fumarate at pH 6·35; ○, starting with sodium L-malate at pH 6·35; △, starting with sodium fumarate at pH 7·3.

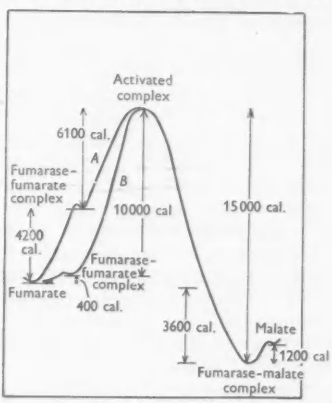

Fig. 13. A diagrammatic representation of the heat changes of the fumarase reaction at pH 6·35. Curve *A* represents the changes occurring above 18°; curve *B* represents those occurring below 18°.

than five times the Michaelis constant concentration, and mostly considerably more. Hence the energy of activation values obtained from these results may be considered as due to the activation of the Michaelis complex. According to the transition state theory of Eyring & Stearn (1939) the heat change of activation is related to the energy of activation as calculated from the Arrhenius equation in the following manner.

$$\Delta H^* = E - RT.$$

Applying this equation to the activation energy values shown in Figs. 3 and 6 we obtain a very good balance of the heat changes associated with the enzyme reaction. Thus at pH 6·35 we have the following ΔH values. Malate has a ΔH value of 3600 cal. below fumarate. The formation of the malate-fumarase complex results in the evolution of 1200 cal. while the activation of the malate-fumarase complex requires 15000 cal. This would bring the malate-fumarase activated complex to 10200 cal. above the level of fumarate. Starting with fumarate we have two temperature ranges to consider. Below the critical temperature of 18° the formation of the fumarate-fumarase complex requires 400 cal. while the activation of this complex requires 10000 cal., a total of 10400 cal. At temperatures higher than 18°, 4200 cal. are required for the formation of the fumarate-fumarase complex, and 6100 cal. for its subsequent activation, a total of 10300 cal. Fig. 13 shows diagrammatically the ΔH values for the enzyme reaction under these conditions. These results strongly suggest that the first stage of the enzymic reaction with either substrate is the formation of an enzyme-substrate complex followed by the thermal activation of this complex to an unstable intermediate activated complex.

DISCUSSION

The experiments reported here throw some light on the mechanics of the reaction brought about by fumarase, but leave many points unexplained. Thus while it is shown that the changeover from one activation energy to another around 18° at low pH values is coupled with a corresponding and compensating change in the heat of formation of the fumarase-fumarate complex, no explanation of why this change should occur is obvious.

It is tempting to believe that such a change is associated with the orientation of water into the reaction, as it occurs only with the hydration reaction and not with the dehydration reaction. Very suggestive circumstantial evidence exists for this explanation; all the similar cases previously described, in which there is a changeover from a high activation energy to a low one as the temperature is increased, have occurred in enzymes associated with hydrolysis. Thus Sizer & Josephson (1942) have shown that invertase, trypsin and pancreatic lipase all have a critical temperature of 0°, above which the activation energy is less than at temperatures below this point. They also show that under certain conditions urease has a critical temperature of 22°, where the same type of phenomenon occurs.

If these changes were associated with a fundamental property of water which changes at a particular temperature, then the critical temper-

ature should be the same with all the enzymes in which this type of effect occurs. This is not so, although it might be possible to divide these enzymes into two groups, one in which the critical temperature is about 0° and the other where the critical temperature is about 20°. Another possibility for the fumarase reaction is that the water is required in the form of its ions and at low pH values the concentration of hydroxyl ions might be limiting at low temperatures. However, if this were so the critical temperature should vary greatly with pH.

It is interesting to note that the solubility of many hydrocarbons in water shows a minimum at 18° (Bohon & Claussen, 1951). It is possible that at low pH values the chemical structure of the enzyme-substrate complex, where the molecule of water is required for the hydration to occur, is hydrocarbon in nature.

The change from a low activation energy to a high activation energy as the temperature is increased, the phenomenon shown at high pH values, seems to be of a different kind from the converse reaction which we have been discussing. Here the break is obtained with both fumarate and L-malate as substrates and, unlike the type shown at low pH values, the critical temperature varies markedly with pH. No conclusive evidence has yet been obtained to explain this type of effect, but some preliminary experiments suggest that at high pH values fumarate dissociates into smaller molecular weight units as the temperature is increased. If this is so the observed effects are easily explainable if the dissociated and undissociated molecules have different temperature coefficients of activity.

This interpretation is perhaps supported by the fact that at pH 7·9 an overall picture of the enzyme reaction like that shown in Fig. 13 can be obtained at low temperatures, but not at high temperatures. Starting with fumarate at a ΔH level of 3600 cal. above malate the heat of formation of the Michaelis complex is 4200 cal. and at temperatures up to 27° the heat of activation is 4300 cal., a total of 12 100 cal. The heat of formation of the malate-enzyme complex is -1200 cal. and the heat of activation is 12 600 cal., a total of 11 400 cal. At temperatures higher than 27°, however, the heat of activation of the enzyme-fumarate complex is 10 200 cal., and all the other heat changes are the same as at lower temperatures. Thus the activated complex starting with fumarate is 18 000 cal. above malate, while the activated complex starting with malate is 11 400 cal. as before.

This discrepancy can be explained if the following assumptions are made. At temperatures about 27° fumarase dissociates into a different molecular weight unit which has the same Michaelis constants as the undissociated molecule but a higher activation energy. The presence of substrate protects the enzyme from this dissociation, malate being a more efficient protector than fumarate.

The different effects of methanol and ethanol are difficult to explain. Perhaps methanol is capable of replacing water in the fumarase reaction, whereas ethanol owing to its larger size is not. If such a reaction were to occur then presumably the combination of alcohol and the hydrocarbon part of the enzyme or enzyme-substrate complex would not be temperature-dependent the same way as would be expected with hydrocarbon-water combination. However, there is as yet no evidence that methanol is capable of replacing water in the fumarase reaction.

The correlation between enzyme activity and K_m with pH is also very difficult to explain. The correlation is not very strict, for example over the range pH 5·6–6·35 the K_m for fumarate does not change, while the activity changes considerably over the same pH range. Also the change of K_m is not directly proportional to the velocity, over the remainder of the pH range studied, but certainly does tend to vary in the same way. This suggests that the Michaelis constant is not merely an equilibrium constant defining the relation between enzyme-substrate complex, free enzyme and substrate, but is a measure also of the rate at which the enzyme-substrate complex is converted to the free enzyme and the products of the reaction. In other words the Briggs & Haldane (1925) modification of the theory of Michaelis & Menten (1913) may be true at least partially for fumarase. However, the evidence presented for the change of K_m with temperature when the pH is kept constant shows that there is no such correlation between K_m and the velocity of the overall reaction as occurs with change of pH at a particular temperature. On the contrary, if it is assumed that the Michaelis constant is an equilibrium constant relating enzyme-substrate complex, free enzyme and substrate, only then is one justified in calculating the heat of formation of the enzyme-substrate complex from the variation of K_m with temperature. When this assumption is made the highly convincing picture of the heat changes of overall reaction shown in Fig. 13 is obtained. One is thus left with the conclusion that one, or perhaps both, of these paradoxical sets of results is fortuitous. It seems more likely that the tendency of K_m to vary with pH in the same way as the maximum velocity is the fortuitous one. There is considerable evidence (to be published later) that the combination of fumarase and substrates is largely ionic in nature. If the same ionic groups responsible for the combination of enzyme and substrate also contribute to the activation of the enzyme-substrate complex, then it follows that there will be a correlation between the value of the Michaelis constant and the velocity of the enzymic reaction, but without the Michaelis constant being dependent on the velocity.

SUMMARY

1. The Michaelis constants of fumarase for its two substrates vary with pH in the same way as the initial velocities of enzyme reaction.

2. The effects of temperature on the rates of the hydration and dehydration reactions of fumarase at various pH values are described and activation energies are calculated. The activation energies of both reactions vary with pH, and for the hydration reaction vary markedly with temperature. At pH values above the optimum, the activation energy increases at a critical temperature, which varies with the pH, as the temperature is increased. At pH values around the optimum there is no variation of activation energy over the temperature range investigated. At pH values lower than the optimum the activation energy decreases at a critical temperature of 18°, which is independent of the pH, as the temperature is increased.

3. The effect of temperature on the Michaelis constants of fumarase at various pH values is described, and the heats of formation of the enzyme-substrate complexes are calculated. The formation of the fumarase-malate complex is exothermic, while the formation of the fumarase-fumarate complex is endothermic. Moreover, the heats of formation of the fumarase-fumarate complex at low pH values change at 18° in such a way as to compensate for the changes in activation energies that occur under similar conditions at this temperature.

4. The variation of equilibrium constant with temperature is described.

5. An overall picture of the heat changes associated with the action of fumarase is proposed.

6. The significance of these results is discussed.

I am greatly indebted to Dr M. Dixon, F.R.S., for designing the temperature control apparatus used in this work. I also wish to express my appreciation to Dr M. Dixon, Dr E. C. Webb, Dr G. Weber, Mr K. Gibson and the other members of the sub-department of Enzyme Biochemistry for their interest in this work and the many helpful discussions which it has involved. The work was carried out on a grant from the Commonwealth Scientific and Industrial Research Organization, Australia.

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The Electrophoresis of Acid Mucopolysaccharides on Filter Paper

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Electrophoresis, using strips of filter paper as a matrix to support the buffer solutions, has frequently been used to separate individual proteins from mixtures: e.g. serum (Durrum, 1950; Cremer & Tiselius, 1950; Wieland & Fischer, 1948; Grassman, Hannig & Knedel, 1951); enzymes (Wallenfels & Pechman, 1951) and artificial mixtures of purified proteins (Kunkel & Tiselius, 1951). Nucleotides have also been separated by means of a modification of this method (Smellie & Davidson, 1951).

The present paper deals with the application of the method to the separation of some acid mucopolysaccharides. It was hoped that it would enable relatively small amounts of material to be examined and that it might give an indication of the amount and nature of any impurities. Blix (1940) showed that, in free solution at pH 8·0, hyaluronic acid from synovial fluid had an electrophoretic mobility of -12 cm.^{2}V.$^{-1}$sec.$^{-1} \times 10^{-5}$ and that chondroitin sulphate from cartilage had a mobility of -17 cm.^{2}V.$^{-1}$sec.$^{-1} \times 10^{-5}$. According to Jorpes (1946) the mobility of heparin preparations was -17 to -19 cm.^{2}V.$^{-1}$sec.$^{-1} \times 10^{-5}$ over the pH range 2–8, and that of chondroitin sulphate was -16 cm.^{2}V.$^{-1}$sec.$^{-1} \times 10^{-5}$. These values suggest that using electrophoretic methods chondroitin

sulphate could be separated from hyaluronic acid but not from heparin. Recently, Gardell, Gordon & Åqvist (1950), using Hyflo Super-Cel slabs moistened with buffer, achieved the electrophoretic separation of hyaluronic acid from chondroitin sulphate.

METHODS AND MATERIALS

Apparatus. Initially apparatus of the type described by Durrum (1950) was used, the length of the filter-paper strips not immersed in buffer being about 24 cm. and the height of the apex of the filter paper above the buffer level being 11 cm. Later experiments with extracts of skin showed that this apparatus was not big enough to allow migrations of sufficient distance to effect good separations. An apparatus in which the filter paper was supported in a horizontal position was then made. It was similar to that described by Grassman *et al.* (1951) except that owing to the greater length of the filter-paper strips (54 cm.) a number of lateral supports of Perspex were incorporated in the box containing the filter paper, and the filter paper was stretched as much as possible so as to prevent undue sagging between the supports. Large vessels were used to hold the buffer solutions, the levels being accurately equalized by means of a siphon which was removed while the current was running. No precautions were taken to protect the ends of the filter paper from pH changes at the electrodes. All the experiments were carried out at room temperature.

Acid mucopolysaccharide preparations. Except where otherwise stated, the acid mucopolysaccharide preparations were as follows. Hyaluronic acid, as supplied, had been prepared from acetone-dried human umbilical cords by extracting with water and precipitating from the extract with ethanol saturated with potassium acetate. The water-soluble material from this precipitate was reprecipitated with ethanol saturated with potassium acetate. It contained 4·30% N; 31·9% hexosamine and 1·6% S. Chondroitin sulphate, prepared from bovine trachea by the method of Bray, Gregory & Stacey (1944), contained 4·72% N and 23·2% hexosamine. Heparin, prepared commercially from ox lung tissue as described in the British Pharmacopoeia monograph, contained 4·01% N and 23·8% hexosamine.

Filter paper and its preparation. Either Whatman no. 2 or 3 MM filter paper was used. Being the more absorbent of the two, 3 MM paper was used when dealing with large amounts of dilute acid mucopolysaccharide solutions. The width of the filter-paper strips varied in accordance with the amount of material used and the method followed in detecting the acid mucopolysaccharides. When these were detected by staining, strips of 2 cm. width were employed; when the detection was by elution and subsequent colorimetric determination, strips 5 cm. wide or more were used.

The acid mucopolysaccharides were usually dissolved in water and put on the filter paper as a thin band at the origin, which was marked with pencil, so as to come about 5 cm. inside the cathode end of the box. Where more than one application was needed, the solution on the paper was dried off, using gentle heat or, in the case of degraded samples, in an electric oven at about 100°. Durrum's (1950) own procedure was followed when using his apparatus. When the horizontal apparatus was used the anode end of the paper was first dipped in the buffer solution so as to wet it up to the point where the acid mucopolysaccharides had been applied. The excess buffer was then blotted off, and the paper placed in the apparatus.

The anode end of the paper was next dipped into the buffer in the anode compartment. After 10–15 min. the buffer had flowed along the paper sufficiently to moisten it up to and just past the origin. The cathode end was then dipped into the cathode compartment buffer. Equilibrium was established in about 15 min. The movement of the acid mucopolysaccharides due to buffer flow was followed by duplicating the procedure on an extra strip of paper using a drop of a 4% acid fuchsin solution instead of the acid mucopolysaccharide solution. The acid mucopolysaccharides could be moved back to the origin by temporarily raising one end of the box to produce a slight buffer flow (Kunkel & Tiselius, 1951). The acid fuchsin also served to indicate approximately the distance of migration of the chondroitin sulphate during electrophoresis (Gardell *et al.* 1950).

In the Durrum apparatus a potential gradient of approximately 5·0 V./cm. was applied for 3–4 hr., and in the horizontal apparatus 3·5–4·0 V./cm. for about 16 hr. After separation had been effected the papers were removed and dried at 110°.

Detection and estimation of the acid mucopolysaccharides. A number of staining procedures were tried in order to show the position of the acid mucopolysaccharides after migration on the filter paper but no really satisfactory method was devised. Either the filter paper adsorbed the dye used (e.g. toluidine blue and mucicarmine) or, being a polysaccharide, reacted in the same manner as the acid mucopolysaccharides (e.g. the periodic acid fuchsin stain). The metachromatic stain with toluidine blue or azure A was the least unsatisfactory. The dried papers were dipped into the toluidine blue solution (0·125%, w/v) or azure A (0·05%, w/v), quickly withdrawn, and blotted. The sulphated acid mucopolysaccharides were easily seen as reddish bands on a blue background. Hyaluronic acid was less easily distinguished. With highly polymerized specimens a reddish colour could be seen, but depolymerized specimens showed only a deepening of the blue background and in small amounts were invisible. Whatman no. 2 filter paper gave better results than 3 MM. As little as 25 μg. chondroitin sulphate and heparin could be seen on strips 2 cm. wide.

The most satisfactory method of detecting the positions of acid mucopolysaccharides was to elute them quantitatively from 1 cm. wide segments cut from the dried filter paper and then to determine the hexosamine or hexuronic acid content of each eluate.

Elution was carried out in the apparatus shown in Fig. 1. The strip of filter paper to be eluted was suspended by copper or nichrome wire at the lower end of the condenser. The acid mucopolysaccharides on the filter paper were completely extracted after refluxing for about 2·5 hr. An oil bath fitted with a test-tube rack enabled twenty extractions to be made simultaneously. The eluate was then evaporated to dryness under reduced pressure and the residue extracted once with 10 ml. of ether to remove any paraffin oil that might have entered the apparatus. Subsequently either hexosamine determinations were made using the Morgan and Elson reaction as modified by Johnston, Ogston & Stanier (1951), or hexuronic acid determinations using the carbazole method of Dische (1947). The former method was the more satisfactory, but filter paper which contained no acid mucopolysaccharides gave an eluate which evoked a slight response on the colorimeter due to a non-specific yellow colour. With the Dische method this non-specific effect was more marked, and in addition charring occurred if any trace of filter paper was washed into the test tube as a result of the

elution. The final colours that were obtained with both methods were read in a Unicam D.G. spectrophotometer, and the optical densities obtained were used as the ordinates in Figs. 3–5.

RESULTS

Experiments using the Durrum apparatus

Fig. 2 illustrates the appearance of the filter-paper strips after electrophoresis and staining in 0·125% (w/v) toluidine blue. Approximately 50 μg. of each acid mucopolysaccharide were subjected to electrophoresis for 4 hr. with a potential gradient of 5 V./cm. on 2 cm. wide strips of no. 2 Whatman filter paper. Sodium phosphate, 0·1 M (pH 6·7), was used as the buffer. The strip of filter paper on which acid fuchsin was placed is illustrated to show that the heavy red band migrated at approximately the same rate as chondroitin sulphate. From these results it would seem that the hyaluronic acid contains an impurity which migrates at about half the rate of chondroitin sulphate, and that the chondroitin sulphate and heparin contained no gross impurities. Although the heparin migrated at a slightly faster rate than the chondroitin sulphate they were not resolved when mixed.

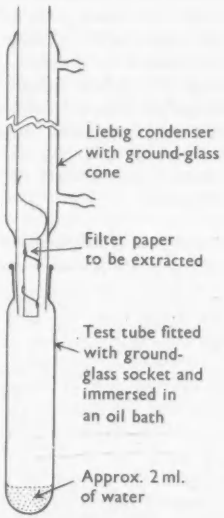
Liebig condenser with ground-glass cone
Filter paper to be extracted
Test tube fitted with ground-glass socket and immersed in an oil bath
Approx. 2 ml. of water

Fig. 1. Apparatus for elution of acid mucopolysaccharides from filter-paper strips.

In Fig. 3 are shown the results obtained by eluting the filter-paper strips after electrophoresis and determining the hexuronic acid content of the eluates. The amounts of acid mucopolysaccharides used and conditions of electrophoresis were the same as those for Fig. 2. Here the impurity in the hyaluronic acid does not appear to be separated from the hyaluronic acid, but the assymetric form of the curve suggests an impurity with a greater mobility than the hyaluronic acid. On the other hand, chondroitin sulphate and heparin both have slower-moving components which did not appear in the strips stained with toluidine blue.

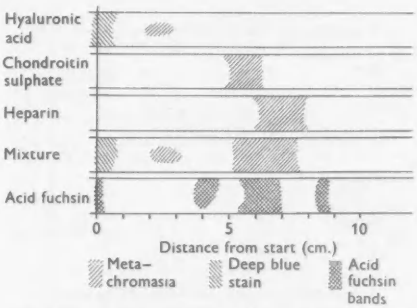

Fig. 2. The appearance of the filter-paper strips after electrophoresis of acid mucopolysaccharides in 0·1 M-sodium phosphate buffer at pH 6·7 and staining with 0·125% (w/v) toluidine blue.

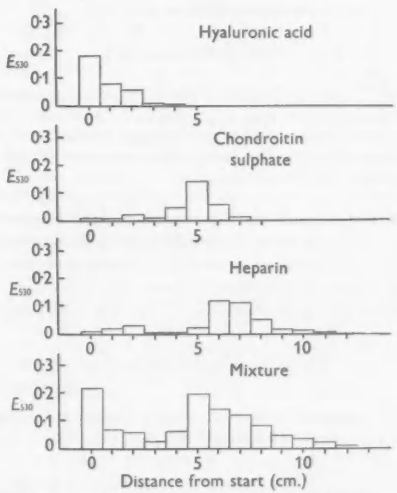

Fig. 3. The positions of the acid mucopolysaccharides as shown by elution of the filter paper and hexuronic acid determination (ordinate, optical densities at 530 mμ.). The experimental details were the same as for Fig. 2.

These results indicate that the technique of filter-paper electrophoresis can resolve mixtures of acid mucopolysaccharides with largely differing mobilities (e.g. hyaluronic acid and chondroitin sulphate) but that the method is not sensitive

enough to separate a substance from another of slightly different mobility (e.g. heparin and chondroitin sulphate were not separated). It can be seen that elution of strips and chemical determination of a component of the acid mucopolysaccharides in the eluate was superior to the staining technique. The Durrum apparatus was discarded for the horizontal apparatus owing to the difficulty in obtaining satisfactory results from extracts of skin even with increased electrophoresis time.

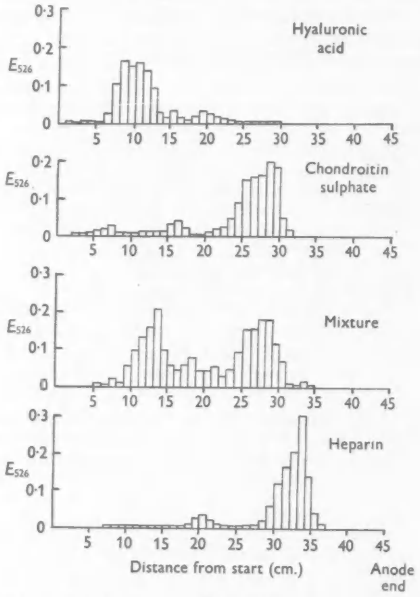

Fig. 4. The electrophoresis of the acid mucopolysaccharides in the horizontal type apparatus in 0·1 M-sodium acetate buffer at pH 5·0. Ordinates represent optical densities at 526 mμ. found in hexosamine determinations carried out on the eluates.

Experiments using the horizontal apparatus

In Fig. 4 are shown the results obtained using the horizontal apparatus. Approximately 1 mg. quantities of each of the acid mucopolysaccharides were placed on 5 cm. wide strips of no. 3 MM filter paper and electrophoresis carried out for 16 hr., in a 0·1 M-sodium acetate-acetic acid buffer, pH 5·0, with a potential gradient of 3·8 V./cm. The positions of migrating substances were shown by eluting strips and making hexosamine determinations on each eluate. There are impurities in all three substances, although the small peak between 5 and 10 cm. from the origin in the chondroitin sulphate diagram was not due to the presence of hexosamine but to a non-specific yellow colour. Chondroitin sulphate was separated from hyaluronic acid. The rate of migration of chondroitin sulphate was not affected by the presence of hyaluronic acid and vice versa. There was no separation of heparin from chondroitin sulphate.

Effect of degree of polymerization on migration of hyaluronic acid

It was noticed that hyaluronic acid, prepared by extraction with alkali from pig skin and rat skin, migrated faster than the umbilical cord hyaluronic acid used in the previous experiments. Owing to differences in the method of preparation of the hyaluronic acid, the samples prepared from skin were greatly depolymerized whilst those prepared from umbilical cord were highly polymerized. To determine whether the degree of polymerization influences the rate of migration of hyaluronic acid, samples of hyaluronic acid from several sources treated in the ways indicated in Table 1, were simultaneously subjected to electrophoresis on separate strips of filter paper in the same apparatus.

Table 1. *The distances of migration of hyaluronic acid from various sources before and after treatment with* N-NaOH

(The electrophoresis was conducted on 3 MM Whatman filter paper in 0·1 M-sodium acetate buffer, pH 5·0, for 17 hr. with a potential gradient of 3·8 V./cm.)

Source of hyaluronic acid	Treatment	State of solution	Distance of migration (cm.)
Bovine synovial fluid	Protein removed with trypsin and adsorption on Lloyd's reagent	Highly viscous	3–9
Bovine synovial fluid	As above and then incubated in N-NaOH at 37° for 48 hr.	Non-viscous	23–28
Umbilical cord	Umbilical cords extracted with cold water and hyaluronic acid precipitated with ethanol	Viscous	9–13
Umbilical cord	As above and then incubated in N-NaOH	Non-viscous	24–29
Pig skin	Prepared by method of Meyer & Chaffee (1941) i.e. initial extraction with N-NaOH at 37° for 48 hr.	Non-viscous	23–27

The results indicated that treatment with alkali increased the rate of migration markedly.

There seem to be two possible explanations for this increased rate of migration. Meyer (1951) showed that undegraded samples of hyaluronic acid possessed fewer acidic groups than degraded samples, as judged by the ash content of their salts. This indicates that degraded samples have a greater charge/weight ratio than undegraded samples, and degraded samples would consequently be expected to have a greater electrophoretic mobility than undegraded samples of hyaluronic acid. Unfortunately the effect of polymerization on the electrophoretic mobility in free solution does not seem to have been studied.

The sample of hyaluronic acid that had not been degraded by alkali formed viscous solutions. The migration of these viscous solutions may have been mechanically obstructed by the filter paper. On two occasions the filter-paper strips were allowed to touch the lid of the apparatus so that a layer of buffer was held between the paper and the lid. The results obtained by elution and glucosamine determination showed very diffuse peaks. These would have been unsatisfactory for most purposes, but they implied that in these circumstances, the rates of migration of hyaluronic acid from umbilical cord in the undegraded and degraded states did not differ greatly. Thus on one occasion the undegraded sample showed a peak at 19–24 cm. from the origin, whereas the alkali-treated samples had a peak at 26–33 cm. (cf. Table 1 where values of 9–13 cm. and 24–29 cm. respectively are given). Presumably the hyaluronic acid was able to migrate in the buffer solution between the paper and the lid of the box at a rate approaching that in free solution. Nevertheless, the degraded sample still migrated faster than the undegraded sample. It is concluded that both the factors mentioned above contribute to the increase in velocity of migration of hyaluronic acid on depolymerization.

Examination of glucosamine-containing compounds obtained from skin

Meyer & Chaffee (1941) prepared both hyaluronic acid and chondroitin sulphuric acid from pig skin. The hyaluronic acid was said to be sulphate-free whereas the chondroitin sulphate fraction was contaminated with some material, presumably hyaluronic acid, which was removed by incubation with pneumococcus hyaluronidase. Meyer & Chaffee's method was used to prepare similar fractions which were examined by electrophoresis in the horizontal apparatus. Amounts equivalent to approximately 10 g. of skin were placed on 5 cm. wide strips of no. 3MM Whatman filter paper and electrophoresis was carried out for 16 hr. at a potential gradient of 4·0 V./cm. in 0·1 M-sodium acetate-acetic acid buffer, pH 5·0. The positions of acid mucopolysaccharides were determined by elution of the strips and hexosamine determination. The results obtained are presented in Fig. 5.

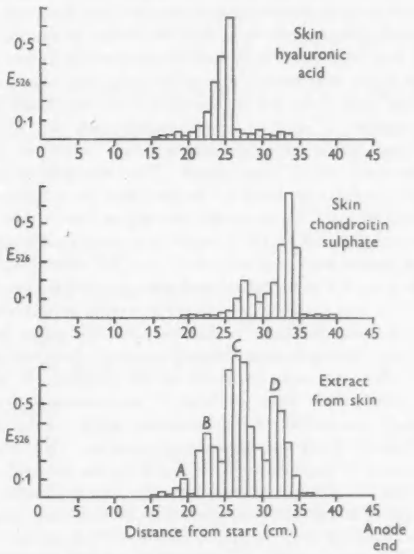

Fig. 5. The electrophoresis in 0·1 M-sodium acetate buffer at pH 5·0 of various skin preparations, each representing material obtained from approximately 10 g. skin. The acid mucopolysaccharides were detected by elution of the strips followed by hexosamine determination. (Ordinate, optical densities at 526 mμ.)

The hyaluronic acid appears to be free of any contaminant that has a different mobility and contains hexosamine, whereas the chondroitin sulphate contains an appreciable amount of an impurity with a mobility similar to that of hyaluronic acid. No attempts were made to remove this impurity by incubation with pneumococcus hyaluronidase.

In Fig. 5 are also shown the data obtained under the same experimental conditions from an alkaline extract of approximately 10 g. pig skin, the proteins having been removed by treatment with Sevag's reagent and adsorption on Lloyd's reagent at pH 5·0. After incubation of the extract with pneumococcus hyaluronidase peaks *A* and *B* were no longer present. Peaks *C* and *D* can each be split into two fractions on the basis of solubility in 10 % (w/v) barium acetate. The barium acetate-soluble fraction of peak *D* is chondroitin sulphate. A detailed presentation of these results will be given later.

DISCUSSION

Electrophoresis of acid mucopolysaccharides can be carried out on filter-paper strips in a manner similar to that used for proteins, amino-acids and nucleotides. Unfortunately, the detection of the positions of the acid mucopolysaccharides on the filter paper after electrophoresis is not satisfactory. There is no staining technique that will show the positions of all the acid mucopolysaccharides on the paper as distinctly and sensitively as for proteins and amino-acids. The stains which are used to demonstrate the presence of acid mucopolysaccharides in histological tissue sections either react with, or are adsorbed on to, filter paper. Thus the acid mucopolysaccharides have to be detected on a coloured background. Unsuccessful attempts were made to use substitutes for filter paper or to modify it so that the stains were not adsorbed etc., for example, by using woven glass fabric and acetylated filter paper.

The acid mucopolysaccharides could be detected after electrophoresis by cutting the filter paper into strips, eluting them and determining the hexosamine or hexuronic acid contents of the eluates. It was possible with this method to demonstrate even small amounts of substances with mobilities different from the main components. The exact amount of such impurities could not be determined owing to the presence of small amounts of substances which were extracted from the filter paper and reacted with reagents used to determine hexosamine or hexuronic acids. For example the first peak in the electrophoresis diagram for chondroitin sulphate in Fig. 4 was not due to a hexosamine-containing substance and was not always present. The second peak was always present. A comparison of Fig. 3 with Fig. 4 shows that the contaminant in the hyaluronic acid preparation contained both hexosamine and hexuronic acid. As it stained metachromatically with toluidine blue it could possibly be sulphated and account for the 1·6 % sulphur in the hyaluronic acid preparation.

All the substances that could be present in acid mucopolysaccharide preparations were not detected since the tests used only detected substances containing either hexosamine or hexuronic acid. Consequently, even if an acid mucopolysaccharide preparation appears to migrate as a single peak in filter-paper electrophoresis, substances for which no tests were made, such as proteins or glycogen, might still be present. The protein content of any acid mucopolysaccharide preparation can be determined by total nitrogen and hexosamine analyses prior to electrophoresis. As many proteins contain hexosamine (up to 2–3 %), acid mucopolysaccharide preparations, containing appreciable amounts of protein, may show on electrophoresis a number of peaks if hexosamine analyses are used to detect the positions of the migrating substances. In such preparations hexuronic acid analyses would show the positions of the acid mucopolysaccharides with greater specificity.

Asymmetric peaks were obtained from mixtures of substances with differing electrophoretic mobilities which did not separate from one another in the course of electrophoretic migration. This asymmetry was more marked when the substances were not present in equal amounts. For example, a highly asymmetric hyaluronic acid peak is shown in Fig. 3, but Fig. 4, which embodies results obtained from a longer migration, shows the main hyaluronic acid peak separated from two smaller peaks. Similar results were obtained from the chondroitin sulphuric acid fraction, prepared from skin by the method of Meyer & Chaffee (1941).

The degree of polymerization of hyaluronic acid affects its rate of migration, and Blix & Snellman (1945) showed that hyaluronic acid was polydisperse. Nevertheless, it does not seem likely that the spread of polymer size in any sample of hyaluronic acid would be sufficient to cause migration rates to differ enough to account for an asymmetric peak. The hyaluronic acid samples listed in Table 1 were either highly polymerized or greatly depolymerized. They represent differences in degree of polymerization far greater than would be expected in any one hyaluronic acid sample.

With the horizontal apparatus described here it does not seem possible to determine electrophoretic mobilities accurately. Evaporation and subsequent alterations in buffer concentration along the filter-paper strips would render any estimations of mobility made in this apparatus of doubtful value.

SUMMARY

1. A horizontal type of apparatus for carrying out paper electrophoresis is described.

2. The electrophoretic migration of three acid polysaccharides, hyaluronic acid, chondroitin sulphate and heparin were examined on filter-paper strips.

3. The positions of the acid mucopolysaccharides after electrophoresis were ascertained by staining with toluidine blue or by quantitative elution from segments of filter paper followed by determination of either the hexosamine or hexuronic acid content of each eluate. An apparatus for the quantitative elution of the acid mucopolysaccharides is described.

4. Extracts of skin have been examined by this method.

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Studies in the Bile Acids

1. A METHOD OF SEPARATION AND IDENTIFICATION

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In order to study the occurrence and metabolism of the bile acids, it is necessary to have methods of identifying the constituents of a mixture. Mixtures of bile acids are difficult to handle by classical techniques, which depend on the formation of characteristic crystalline derivatives. Frequently, even relatively pure compounds are not easy to crystallize, and large amounts of material are necessary if minor constituents are to be identified. As pointed out by Haslewood & Wootton (1950), much previous work is not based on acceptable experimental evidence.

In recent studies of steroid hormones and their metabolites, considerable use has been made of chromatographic separation combined with infrared spectrometry (Dobriner, Lieberman & Rhoads, 1948*a*; Dobriner *et al.* 1948*b*; Lieberman & Dobriner, 1948; Jones & Dobriner, 1949). In combination, these techniques have provided a powerful method for separating and identifying the individual non-acidic steroids of complex mixture. It therefore seemed probable that a similar method could be applied to the bile acids.

A successful procedure is described here. Initial trials with pure substances showed that the methyl esters of the common bile acids are separable on silica gel. These esters are soluble enough to enable their specific infrared spectra to be recorded in carbon disulphide solution.

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EXPERIMENTAL

Solvents. All solvents except ether were of 'C.P.' quality and were redistilled before use. Absolute ether was passed through a column of Al_2O_3. Care was taken to use the purest solvents available, otherwise traces of unknown impurities tended to obscure parts of the spectrum. For this reason, a mixture of equal parts of pentane and hexane ('P.H.') (American Mineral Spirits Co., New York) was used as a solvent, with properties similar to those of light petroleum. Proportions of all solvent mixtures are v/v.

Chromatography. Columns of dry silica gel were used (Dobriner, personal communication). A column was prepared by filling a suitable tube with P.H. The appropriate quantity (usually 200 times the weight of the esters) of silica gel (grade 923, through 200, Davison Chemical Corporation, Baltimore) was then added in a fine stream and the solvent allowed to flow until a compact column was formed. The esters were applied as a solution in a solvent of relatively low polarity (e.g. P.H., 90:ether, 10), and development proceeded with solvent mixtures of increasing polarity. In a typical chromatograph, the solvent mixtures were: (1) P.H., 99:ether, 1 to dissolve the esters. (2) Mixtures of P.H. with ether containing 5, 10, 15, 20, 30, 40, 60 and 80% ether. (3) Pure ether. (4) Mixtures of ether and acetone, containing 5, 10 and 50% acetone. (5) Pure acetone. (6) Acetone, 50; methanol, 50. (7) Pure methanol.

Amounts of 100–250 ml. of each solvent mixture were applied, the eluate being collected in 50 ml. fractions at a rate of 2–3 drops/sec. If necessary, N_2 under pressure was admitted to the solvent reservoirs to maintain this rate of flow. Each fraction was taken to dryness at once and, if eluted material was present, the same solvent mixture was continued until no significant residue was found. The next solvent mixture was then applied.

Infrared spectrometry. The residues from all the individual fractions were dissolved in CS_2 and examined in the spectrometer. The instrument used was a Perkin-Elmer model 12, fitted with a sodium chloride prism and a 3 mm. cell of capacity 0·03 ml. (Colthup & Williams, 1947; Hardy, Wilson & Dobriner, 1949). With a cell of this capacity, about 100 μg. of ester were required to produce a well defined spectrum. Fractions containing a larger quantity were diluted appropriately.

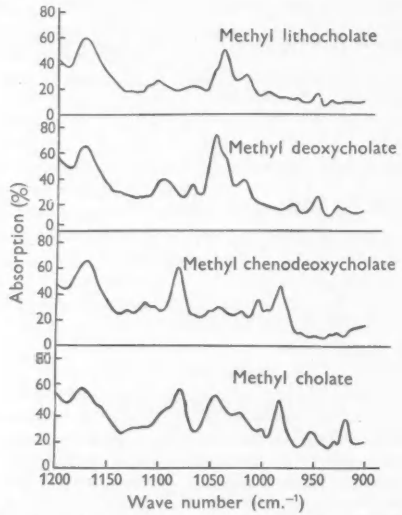

Fig. 1. Infrared absorption spectra of bile acid esters in CS_2 solution. The spectra cover the region of the molecular 'finger-print'.

The spectra were regularly recorded over the frequency range between 1180 and 875 cm.$^{-1}$. In this 'finger-print' region, all the many steroids so far examined show marked individuality (Jones & Dobriner, 1949), including the bile acids (Fig. 1). Whenever necessary, other regions of the spectrum were also recorded.

Melting points. A hot-stage microscope was used. All melting points are corrected.

Experiments with pure compounds

Experience in the separation procedure was gained by chromatographing mixtures containing the methyl esters of lithocholic acid (3-hydroxycholanic acid), deoxycholic acid (3:12-dihydroxycholanic acid), chenodeoxycholic acid (3:7-dihydroxycholanic acid) and cholic acid (3:7:12-trihydroxycholanic acid). It was found that these compounds fell into three well separated groups. Methyl lithocholate was eluted from the column first, followed by the two dihydroxy esters which were recovered as a mixture. Finally, pure methyl cholate was obtained.

Attempts were made to separate the dihydroxy esters by using a different solvent system (benzene and ethanol) and by chromatography on alumina. The best results, however, were obtained by acetylating the mixture of dihydroxy esters and rechromatographing the diacetates on silica gel. A fair separation was then possible, with methyl diacetyldeoxycholate being eluted first, but some overlapping usually occurred. The spectra of the binary mixtures were readily identified.

The approximate solvent mixtures required to elute different substances are given in Table 1, which also includes two additional compounds encountered later in this investigation. In different chromatograms, the various compounds always appeared in the same order and there were only slight variations in the mixtures required to elute a given substance.

Table 1. *Solvent mixtures required to elute compounds from silica gel*

Solvent mixture	Compound
Before acetylation	
P.H.*-ether 70:30	Cholesterol
P.H.-ether 40:60	Methyl lithocholate
P.H.-ether 20:80	Unidentified keto acid (not present in ox bile)
Ether	Methyl deoxycholate and methyl chenodeoxycholate
Ether-acetone 50:50	Methyl cholate
After acetylation	
P.H.-ether 85:15	Methyl diacetyldeoxycholate followed by methyl diacetylchenodeoxycholate

* P.H. is pentane-hexane (1:1).

Application to analysis of ox bile salts

Material. This was a preparation made by Abbott Laboratories, Chicago, containing the 90% ethanol-soluble constituents of fresh ox bile.

Hydrolysis. The pale yellow powder (4·1 g.) was dissolved in water (75 ml.) and mixed with a solution of 8 g. NaOH in 15 ml. water. The solution was heated on a steam bath for 20 hr., made acid with 10 N-HCl, saturated with solid NaCl and allowed to stand for some hours. The solid material was washed with water, dried and exhaustively extracted with boiling ethanol. The ethanolic solution yielded 2·5 g. of a brown gum containing the mixed bile acids.

Methylation. The crude acids were dissolved in methanol and treated at 0° with an excess of ethereal diazomethane solution: after evaporation of the solvent, the residue was dissolved in ether. The solution was washed with 0·2 N-NaOH, dried and taken to dryness, leaving the mixed esters as an amber gum weighing 2·3 g.

All subsequent work was done on a portion of the mixed esters weighing 250 mg.

Initial chromatogram. The material (250 mg.) was dissolved in 250 ml. P.H.-ether (99:1) and applied to a column 17 cm. high, 2·2 cm. diameter, containing 50 g. silica gel. Subsequent development produced 102 fractions of 50 ml. each, summarized in Table 2.

Fractions 42–50. The combined material was dissolved in P.H.-methanol and yielded a few crystals, m.p. 118–119° not depressed by mixture with authentic methyl lithocholate, m.p. 123°.

Fractions 80–92. This material crystallized as leaflets from P.H.-ether, m.p. 151–2°, raised by recrystallization

from P.H.-ethanol to 158° unaltered by mixture with methyl cholate, m.p. 157–158°.

Second chromatogram. The material in fractions 56–70 was acetylated at room temperature with 0·1 ml. acetic anhydride and 0·1 ml. pyridine. The product was dissolved in 100 ml. P.H.-ether (95:5) and chromatographed on a column 28 cm. high, 0·7 diameter, containing 8 g. silica gel. Development was carried out with P.H.-ether (85:15), the eluate being collected in 50 ml. fractions. Pure methyl diacetyldeoxycholate was found by infrared spectrometry in fractions 9–22. The combined material crystallized from aqueous ethanol as long needles, m.p. 116–117°.

Fractions 23–30 were combined and weighed 1·5 mg. From its spectrum it was evident that this material consisted of a mixture of methyl diacetyldeoxycholate and methyl diacetylchenodeoxycholate. Accordingly, mixtures containing various proportions of these two esters were prepared and submitted to spectrometry. The spectrum of 60% methyl diacetyldeoxycholate and 40% methyl diacetylchenodeoxycholate matched most closely the spectrum of the combined fractions (Fig. 2). It was concluded that the latter contained 0·6 mg. methyl diacetylchenodeoxycholate and 0·9 mg. methyl diacetyldeoxycholate.

Table 2. *Initial chromatogram of mixed esters from ox bile*

(Fractions were 50 ml. each. All fractions not described contained negligible amounts of material.)

Fractions	Eluant	Description	Identification by infrared spectrometry, etc.
8–11	P.H.-ether 95:5	Trace of white solid	Unknown, negative Pettenkofer reaction
26–31	P.H.-ether 70:30	1·1 mg. waxy solid	Cholesterol
42–50	P.H.-ether 40:60	1·8 mg. colourless gum	Methyl lithocholate
56–70	Ether 100	38·6 mg. colourless gum	Methyl deoxycholate
80–92	Ether 50; acetone 50 and onwards	181 mg. yellow gum	Methyl cholate

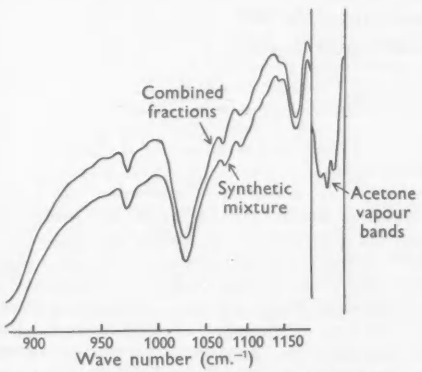

Fig. 2. The infrared spectrum of material isolated from ox bile salts compared with that of a mixture of 60% methyl 3:12-diacetoxycholanate and 40% methyl 3:7-diacetoxycholanate. The spectra are traced from the original record and appear against a sloping background of changing energy. Absorption bands are registered as downward-pointing 'peaks' and a wavelength marker of acetone vapour bands is included.

DISCUSSION

Chromatography has often been found useful for separating mixtures of steroids (for references see Reichstein & Shoppee, 1947). As a method of identifying the separated bile acid esters, infrared spectrometry possesses considerable advantages. Experience with the spectra of steroids has shown that their specificity is beyond question (Jones & Dobriner, 1949). In the work described here, crystalline esters were obtained when possible, but the primary identification was made by examining the spectra, and the mixed melting points were regarded as only confirmatory. Thus a compound like chenodeoxycholic acid, whose derivatives are difficult to crystallize, can be identified as easily as cholic acid.

The amount of material required is well illustrated by the results of the second chromatogram, in which less than 1 mg. of methyl diacetylchenodeoxycholate was found, from the total 250 mg. of mixed esters. No bile acids were encountered other than the four known to be present in ox bile. The keto acids described by Wieland & Kishi (1933) and Haslewood (1946) were not encountered; in view of the minute amounts which these authors isolated, this result was not surprising.

When analysing urinary steroids, Dobriner *et al.* (1948) made use of specific reactions (e.g. the Zimmermann-Callow reaction) to determine the amount of steroid material present in their various fractions. In the absence of such specific reactions, it has been necessary in this work to weigh the fractions as an estimate of the amount of steroid present. The esters are obtained in a fairly pure form, as indicated by their spectra and by the ease with which they crystallize, so that it is considered that the weights form a reasonably accurate estimate. However, it is hoped to develop alternative methods, one possibility being to make absorption measurements of the band at 1742 cm.$^{-1}$ characteristic of the —$COOCH_3$ group.

SUMMARY

1. Chromatography on silica gel, combined with infrared spectrometry, has been used to analyse mixtures of bile acids.

2. The results obtained by applying this method to an ox bile-salt preparation are described. Methyl lithocholate (methyl 3-hydroxycholanate) (1·8 mg.), methyl deoxycholate (methyl 3:12-dihydroxycholanate) (38 mg.), methyl chenodeoxycholate (methyl 3:7-dihydroxycholanate) (0·6 mg.) and methyl cholate (methyl 3:7:12-trihydroxycholanate) (181 mg.) were recovered from 250 mg. of crude mixed esters.

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Enzyme inhibitions by Snake Venoms

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Snake venoms are complex mixtures of components, many of which are known to be proteins having enzymic activities. Their enormous toxic power is illustrated by the fact that the minimum lethal dose for mice of a purified preparation of neurotoxin from cobra venom is 9·0–0·03 μg./g. weight of animal (Micheel & Jung, 1936). The complex nature of venoms gives rise to a variety of symptoms whose dominant clinical features have caused the venoms to be classified as neurotoxic, haemotoxic or depressant.

Although the presence of proteolytic enzymes in venoms was described by de Lacerda (1881), it was only after an investigation of the coagulating effect of some varieties of venoms (viperine) (Lamb, 1901; Noc, 1904; Martin, 1905) had stimulated the study of venom proteases, and after Delezenne & Ledebt (1911*a*, *b*) had correlated the presence of lecithinase with haemolysis, that it became evident that enzymes may play a significant role in the pathogenic effects of venoms. Feldberg & Kellaway (1937, 1938) have pointed out that the effects of cobra venom on the circulatory system may be accounted for, to some extent, by the presence of a phosphatidase.

A considerable variety of enzymes is now known to exist in snake venoms. These are proteolytic enzymes, phosphatidases, hyaluronidase, amino-acid oxidase, phosphoesterases, 5-nucleotidase, ribonuclease and deoxyribonuclease, cholinesterase, adenosinetriphosphatase and diphosphopyridine nucleotidase. (For review see Zeller, 1951.) How far these enzymes, or other components of venoms, are implicated in the production of neurological lesions by venoms is still quite obscure.

The variety of symptoms produced by venoms on the nervous system has been attributed to components loosely referred to as 'neurotoxins'. Most investigations have pointed to the protein nature of neurotoxins. A long time ago Mitchell (1860) separated a protein fraction from rattlesnake venom which was toxic to pigeons. Slotta & Fraenkel-Conrat (1938) reported the isolation of a neurotoxin of *Crotalus terrificus terrificus* in crystalline form which they termed 'crotoxin'. This protein has been found to possess phosphatidase activity and to

be homogeneous by electrophoresis (Li & Fraenkel-Conrat, 1942) and by the ultracentrifuge (Gralén & Svedberg, 1938).

The object of the present investigation was to discover whether the neurotoxic effects due to cobra venom might result from a disturbance of enzyme activities in the nervous system. There are, however, so many factors in venom capable of influencing its course of action that it is difficult to assess which, if any, of these factors is essential for toxicity. Investigations by Chain (1939), Ghosh & Chatterjee (1948), Fleckenstein, Tippelt & Kroner (1950) and by Fleckenstein & Gayer (1950) have shown that crude venom of a variety of snakes inhibits the activities of several dehydrogenases and oxidizing systems, but it is difficult to determine which factor is primarily involved in these inhibitions. It has, therefore, been considered necessary, in the work to be described, to treat the venom in such a way that, whilst retaining neurotoxic effects, the problem of interpretation is simplified by removal of a large variety of enzymes.

It has been known since the pioneer studies of Mitchell (1860) that when venom is heated to 100° for 30 min. it still retains toxic properties. A sample of cobra venom (from the Haffkine Institute, Bombay, India) heated for 15 min. in a boiling-water bath and tested for toxicity by intramuscular injections in mice, is lethal. For example, 50 μg. of heated venom in NaCl (0·9%) injected into six mice, killed the animals in 4 hr. Since heating the venom for 15 min. at 100° does not destroy the toxicity of cobra venom to animals, it became important to discover what enzymic activity might still remain in the heated venom.

METHODS AND MATERIALS

The venoms employed were five in number: Russell's viper venom supplied by the Haffkine Institute; Russell's viper venom obtained from Wellcome Research Laboratories, London; *Agkistrodon piscivorus* venom supplied by Ross Allen's Reptile Institute, Silver Springs, Florida; cobra venom (*Naja naja*) obtained from the Haffkine Institute; and cobra venom supplied by Dunning Inc., Baltimore. Most of the work to be described was carried out using cobra venom supplied by the Haffkine Institute. The heated venom consisted of the supernatant obtained after removing the protein coagulated on heating a solution of the crude lyophilized venom in a boiling-water bath for 15 min. The heat-treated product, prepared as described above, on drying, contained 8% N, and therefore had a protein concentration of approx. 50%.

Experiments were carried out to investigate the enzymes of heated and unheated venom and the action of heat-treated venom on the metabolism (respiration, glycolysis) of tissue homogenates. A series of isolated enzyme systems was also examined, using the usual Warburg technique.

L-Amino-acid oxidase. Measurements of O_2 uptake obtained after addition of 0·02 M-L-leucine to solutions of venom in phosphate buffer, pH 7·4, were made (Zeller, 1948).

Cholinesterase. Rates of hydrolysis of acetylcholine on addition to venom dissolved in 0·025 M-$NaHCO_3$ were measured manometrically (Quastel, Tennenbaum & Wheatley, 1936).

Choline acetylase. Experimental details as given by Harpur & Quastel (1949) were followed.

Choline dehydrogenase. Preparations of tissue and determinations of activity were carried out according to Mann & Quastel (1937).

Pyruvic dehydrogenase of brain. Measurements of the activity of this enzyme in a brain homogenate were made with the use of the ferricyanide technique in presence of pyocyanin as carrier (Michaelis & Quastel, 1941). The homogenates were prepared by the following procedure: soon after removal from the animal, the brain was sliced, the slices were well washed in 30 ml. of 0·9% NaCl, and then minced for 1 min. in a small quantity of 0·9% NaCl.

Pyruvic oxidase of pigeon-breast muscle. This enzyme was prepared according to Stumpf, Zarudnaya & Green (1947), and its activity determined by the ferricyanide technique (Quastel & Wheatley, 1938).

Glucose oxidase of acetone-dried pigeon liver. Acetone-dried powders were prepared as follows: six fresh pigeon livers were cut up with scissors and ground in a Waring Blendor with a small quantity of water (50 ml.). The paste was added to ice-cold acetone (20 vol.). The mixture was allowed to stand for about 20 min. and filtered by suction. The powder obtained was further treated with 200 ml. of ice-cold acetone, filtered by suction and dried quickly in air by spreading over filter paper. The dried powder was kept in a vacuum desiccator in the refrigerator. Acetone-dried pigeon-liver powder 500 mg. was extracted with 10 ml. water for 2 hr. in the cold. The extract, after squeezing through muslin, was centrifuged at 3000 rev./min. The clear centrifugate was dialysed overnight at 5° against 400 ml. distilled water. The activity of the glucose dehydrogenase in the dialysate was determined in the presence of added diphosphopyridine nucleotide by the ferricyanide technique (Michaelis & Quastel, 1941). To 1 ml. of dialysed extract of the pigeon liver (equivalent to 50 mg. of the powder) 1 mg. DPN (diphosphopyridine nucleotide) and glucose and $NaHCO_3$ were added equivalent to a final concentration of 0·05 and 0·025 M respectively. Venom was then added to the system which was incubated at 37° in 93% N_2 and 7% CO_2 (v/v) for 60 min. before addition of the ferricyanide from the side arm of the manometric vessel.

Lactic and malic dehydrogenases. These enzymes, in brain homogenates, were examined anaerobically by the ferricyanide technique.

Diphosphopyridine nucleotidase (DPN-ase). The technique of Mann & Quastel (1941) was followed.

Succinic dehydrogenase. The activity of this enzyme was determined by the ferricyanide technique.

Glucose oxidase of fungi (notatin). The technique of Keilin & Hartree (1948) was adopted.

Pyruvic dismutation system. Acetone-dried pigeon-liver powder was used as the source of the enzyme system. The pyruvic dismutase activity was determined manometrically by measuring CO_2 evolved when sodium pyruvate solution was added to a medium containing the enzyme system and $NaHCO_3$ in an atmosphere of 7% CO_2 and 93% N_2 at 37°. The medium contained the following constituents: $NaHCO_3$

(0·025 M); phosphate buffer, pH 7·4 (0·02 M); $MgCl_2$ (0·01 M); DPN (1 mg./vessel); sodium pyruvate (0·02 M); heated venom (300 μg./ml.). When venom was added the system containing the venom and enzyme was incubated for 60 min. before addition of the pyruvate.

Acetylation of sulphanilamide in presence of added adenosinetriphosphate (ATP). Acetone-dried pigeon-liver powder was used. The medium employed, when the acetylation of sulphanilamide was determined in presence of added ATP, contained the following constituents: sodium phosphate buffer, pH 7·4 (0·02 M); $NaHCO_3$ (0·028 M); $MgCl_2$ (0·01 M); NaF (0·02 M); ATP (0·003 M); sodium acetate (0·02 M); sulphanilamide (75 μg./vessel); pigeon-liver powder (50 mg./vessel). The experiment was carried out in manometric vessels in air at 37° for 90 min. The free sulphanilamide remaining at the end of incubation period was determined by the method of Brattan & Marshall (1939).

Acetylation of sulphanilamide in presence of a respiring system. Fresh rat brain was homogenized in a medium having the following composition: $MgCl_2$ (0·006 M); KCl (0·090 M); potassium phosphate buffer, pH 7·4 (0·02 M). Experiments were carried out in manometric vessels at 37° for 90 min. using air as the gas phase. The following constituents were present in each vessel: brain homogenate, 140 mg.; pigeon-liver powder, 75 mg.; sodium pyruvate (0·01 M); sulphanilamide, 80 μg.; heated cobra venom, 300 μg./ml.

Acetylation of sulphanilamide anaerobically in presence of a pyruvate-dismuting system. Experiments were carried out in manometric vessels at 37° in an atmosphere of 7% CO_2 and 93% N_2 for 90 min. The vessels contained the following constituents: Na_2HPO_4 (0·02 M); $NaHCO_3$ (0·025 M); $MgCl_2$ (0·01 M); KF (0·05 M); DPN, 1 mg.; pyruvate (0·02 M); heated cobra venom, 300 μg./ml.; sulphanilamide, 75 μg.; pigeon-liver powder, 75 mg.

Urease. The activity of this enzyme was determined by the manometric assay of CO_2 evolved when a solution of urea placed in the side arm of a Warburg manometric vessel was tipped into a medium containing urease and buffer. The constituents of the flasks were as follows: 0·2 ml. of 10% urea; 0·2 ml. of 400 μg./ml. urease (Dunning) and 0·13 M-acetate buffer (pH 5·0) to 3·2 ml.

When studies of the effect of heated venom on the respiration of tissue homogenates were carried out, a solution of the venom was added to the tissue-substrate mixture immediately after thermal equilibrium had been reached. With studies of the effects of the venom on isolated enzyme systems, the venom was incubated with the enzyme under investigation for a standard time (usually 60 min.), after which the substrate was added.

RESULTS

Activities of enzymes in crude and heated venoms

The activities of cholinesterase, ATP-ase, DPN-ase, L-amino-acid oxidase and lecithinase were estimated in crude and heated snake venoms. The results showed that, although the crude venom contains all the enzymes mentioned, only lecithinase was active in the heated venom, though to a diminished extent. Heating venom at 100° for 15 min. fully inactivates the proteolytic enzymes (Ghosh & Chaudhury, 1938), hyaluronidase (Madinaveitia, 1941), 5-nucleotidase (Zeller, 1950) and phosphatases (Gulland & Jackson, 1938) that are normally present. Taborda, Taborda, Williams & Elvehjem (1952) have recently shown that ribonuclease activity of *Bothrops jararaca* venom is completely lost by heating the venom to 80, 90 and 100°.

It appears probable, therefore, that of all the enzymes known to occur in crude venom only the lecithinase present retains activity after heating at 100° for 15 min.

The effect of heated cobra venom on the oxidation of various substrates by brain tissue

The tissue used was rat-brain homogenate, unless otherwise stated. The brain was removed from the animal soon after decapitation, chilled in cracked ice and homogenized in a Potter-Elvehjem homogenizer for 1 min. in 0·9% NaCl solution. A 1 ml. portion of the homogenate containing 150 mg. brain was used per vessel. The brain homogenate was suspended in phosphate-Locke solution, pH 7·4, containing the following constituents (final concentrations): Na_2HPO_4 (0·02 M); NaCl (0·13 M); KCl (0·004 M); $CaCl_2$ (0·002 M). The substrates were neutralized where necessary. Heated cobra venom, contained in 0·3 ml., was tipped from the side arm of the Warburg manometric vessel after thermal equilibration. O_2 uptakes were measured at 37°. The gaseous phase in the manometric vessels was air. CO_2 was absorbed by KOH in the centre wells.

The results are shown in Table 1. It is evident that heated venom inhibits the rate of oxygen uptake of the brain homogenate in the presence of a variety of substrates. The inhibition increases progressively with time. The substances investigated were glucose, fructose, pyruvate, succinate, L-glutamate and α-ketoglutarate.

The extent of inhibition and the course of the development of the effect differ with different substrates. The results shown in Table 1 give the percentage inhibitions produced by heated venom on the oxygen uptake found with various substrates after 50 and 100 min. contact of venom with brain tissue. When glucose and sodium pyruvate are used as substrates, the heated venom produces an initial small acceleration of respiration of the brain homogenate which falls off with time. This acceleration, which is always found with these substrates, is absent with the other substrates used.

Further results are recorded in Fig. 1 which show that the inhibition of the rate of oxygen uptake by rat-brain homogenate in presence of glucose decreases with diminishing concentrations of heated venom, and the initial activating effects become more pronounced. The inhibition of respiration in presence of fructose starts immediately, but the percentage inhibition produced after 100 min. is no greater than that found for glucose or pyruvate.

In the presence of sodium succinate or sodium L-glutamate the inhibitory effect of heated venom is

apparent almost immediately and increases rapidly. After 100 min. the inhibition of total oxygen uptake was 57 % for succinate and 68 % for glutamate. The oxidation of α-ketoglutarate is only feebly affected by the heated venom, the inhibition after 100 min. being only 16 %. In the absence of added substrate the heated venom produced an immediate fall in respiration which increased with time.

The exact nature of the endogenous respiration of brain is obscure. The pronounced differences obtained between the inhibitory effects of heated venom on glucose and pyruvate oxidations and the inhibition of the residual respiration of the homogenate indicate that glucose and pyruvate are probably not endogenous substrates.

The initial accelerating effect of venom on the oxidation of glucose and pyruvate is diminished in the presence of added ATP (0·01 M), which itself secures an increased rate of respiration. Possibly, the initial acceleration due to venom is caused by the liberation of adenylic acid (or a derivative of this) from the brain tissue by the action of venom. Kellaway & Trethewie (1940) found that the presence of cobra venom brings about the liberation of adenylic acid and its derivatives from rabbit heart and liver.

Table 1. *Percentage inhibition produced by heated cobra venom (300 μg./ml.) on the oxidation of various substrates by rat-brain homogenate*

Exp.	Substrates added to brain homogenate (150 mg.)	After 50 min. O_2 uptake (μl.)	After 50 min. Percentage inhibition of total O_2 uptake	After 100 min. O_2 uptake (μl.)	After 100 min. Percentage inhibition of total O_2 uptake
1	Glucose (0·01 M)	202	—	324	—
	Glucose + heated venom	200	Nil	260	20
	Pyruvate (0·02 M)	218	—	350	—
	Pyruvate + heated venom	198	9	234	33
	Succinate (0·05 M)	350	—	570	—
	Succinate + heated venom	183	48	248	57
	Nil	138	—	206	—
	Nil + heated venom	76	45	90	56·5
2	Fructose (0·02 M)	150	—	285	—
	Fructose + heated venom	115	23	193	32
	α-Ketoglutarate (0·02 M)	109	—	199	—
	α-Ketoglutarate + heated venom	102	6	166	16
	L-Glutamate (0·02 M)	124	—	250	—
	L-Glutamate + heated venom	55	56	90	68
	Nil	90	—	150	—
	Nil + heated venom	70	33	90	40

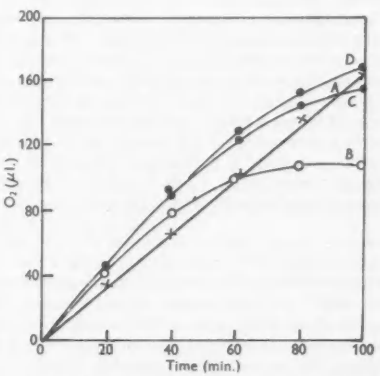

Fig. 1. The effect of different concentrations of heated venom on glucose (0·01 M) oxidation by rat-brain homogenate (100 mg.). Determinations were made as described in the text. *A*, brain + glucose; *B*, brain + glucose + heated cobra venom (100 μg./ml.); *C*, brain + glucose + heated cobra venom (30 μg./ml.); *D*, brain + glucose + heated cobra venom (20 μg./ml.).

The effects of different heated venoms on pyruvate oxidation by guinea pig brain homogenate

Different venoms were tested for their effects on the oxidation of pyruvate by guinea pig brain homogenate in case there was any pronounced difference in behaviour. The four samples tested were cobra venom (Haffkine), cobra venom (Dunning), *Agkistrodon piscivorus* venom, and Russell's viper venom (Haffkine). The first three of these belong to the neurotoxic group of venoms. Russell's viper venom has the least neurotoxic action of the venoms investigated.

The three venoms belonging to the neurotoxic group show only quantitative differences in their behaviour (Fig. 2). They produce the usual initial activation followed by progressively developing inhibition. Cobra venom (Haffkine) is the most potent, cobra (Dunning) is next in potency and the *A. piscivorus* venom has the least effect. In contrast

to the behaviour of the neurotoxic group of venoms, Russell's viper venom brings about an increased rate of respiration throughout the experimental period.

The effect of heated venom on different components of the succinic oxidase system

Since heated venom causes a pronounced inhibition of the succinic oxidase system in brain homogenates, it was of importance to study the action of heated venoms on separate components of the system. Although there may be some dispute regarding the various factors which are believed to be the intermediate links between the dehydrogenases and the cytochrome system, it is established

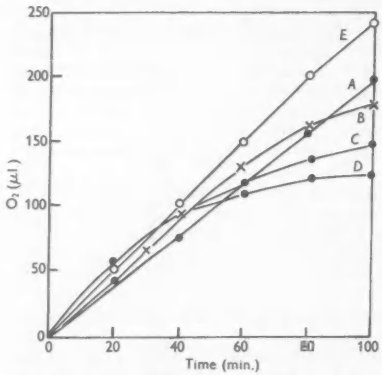

Fig. 2. The effects of different heated venoms (300 µg./ml.) on pyruvate (0·02 M) oxidation by guinea pig brain homogenate (100 mg.). *A*, brain + sodium pyruvate; *B*, brain + sodium pyruvate + *Agkistrodon piscivorus* venom; *C*, brain + sodium pyruvate + cobra venom (Dunning); *D*, brain + sodium pyruvate + cobra venom (Haffkine); *E*, brain + sodium pyruvate + Russell's viper venom.

that the oxidation of succinate is accomplished through the combined action of succinic dehydrogenase, cytochrome *c*, cytochrome oxidase and oxygen. The toxic actions of heated venom on the various components of this system were studied separately. As a result of these studies it was found that venom attacked the succinic oxidase system at more than one point. Both cytochrome oxidase as well as succinic dehydrogenase were found to be inactivated by the heated venom, whilst cytochrome *c* was apparently completely resistant to its effects.

Cytochrome oxidase. Cytochrome oxidase activity was determined using neutralized *p*-phenylenediamine as substrate and rat-brain homogenate as the source of enzyme. It is seen (Fig. 3) that in 1 hr. the venom inhibited 50 % of the activity of the enzyme. In 2 hr. the inactivation of the enzyme was found to be complete. These results are in agreement with the findings of Chatterjee (1949) who has isolated from cobra venom, by salt fractionation, a substance which is inhibitory to cytochrome oxidase.

Cytochrome c. The effect of heated cobra venom on cytochrome *c* was investigated by observing the changes in the spectrum of reduced cytochrome *c* as measured by the

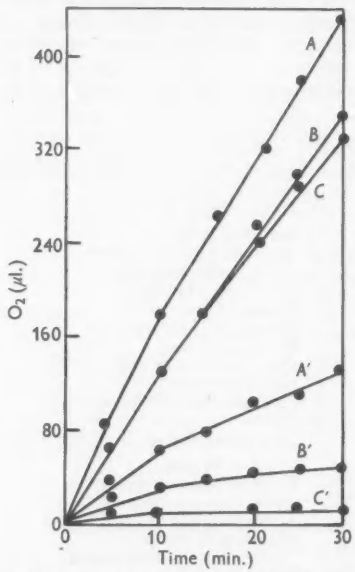

Fig. 3. Variation, with time, of the inhibitory action of heated cobra venom on cytochrome oxidase activity of rat-brain homogenate. The rat-brain homogenate (175 mg./vessel) was suspended in phosphate-Locke (see text). Substrate, *p*-phenylenediamine (20 mg./vessel). Heated cobra venom, 300 µg./ml. Temperature, 37°. Gas phase, air. The substrate was added from the side arm of the Warburg vessel at definite intervals of time after the venom had been in contact with the enzyme. The µl. O_2 absorbed were recorded for 30 min. after adding the substrate. *A*, *B* and *C*, substrate added after 60, 90 and 120 min. respectively. *A′*, *B′* and *C′*, venom present, substrate added after 60, 90 and 120 min. respectively.

Beckman spectrophotometer. A solution of cytochrome *c* in 0·2 M-phosphate, pH 7·4, containing 0·3 mg./ml. was incubated with heated venom prepared in the usual way for 30 min. at 37°. The final concentration of the venom was 300 µg./ml.; 3 ml. of the mixture were then added to the cell and the spectrum determined after reduction with sodium dithionite. The results showed complete identity of the spectra, before and after incubation with heated venom.

The effect of heated venom on succinic dehydrogenase

The activity of succinic dehydrogenase, measured anaerobically by the ferricyanide technique, is greatly reduced on exposure of the enzyme to heated

venom. Typical observations are shown in Fig. 4. These illustrate the relative susceptibilities of the brain and heart-muscle preparations of succinic dehydrogenase to the action of heated venom. The heart-muscle preparation is apparently more vulnerable than the brain preparation.

Determinations were made of the effects of changes in concentration of heated cobra venom on the percentage inhibitions of succinic dehydrogenase

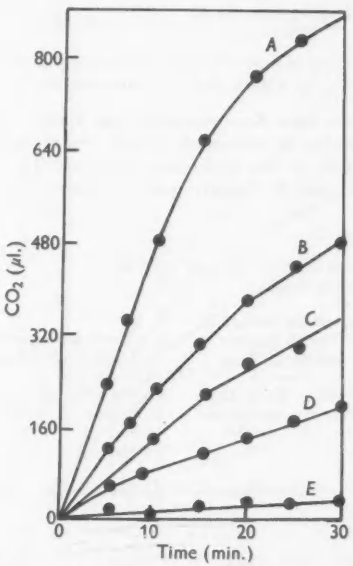

Fig. 4. The effect of heated cobra venom on succinic dehydrogenase activity of rabbit brain and heart tissue homogenates. Medium: bicarbonate-Locke ($NaHCO_3$, 0·025 M; NaCl, 0·3 M; $CaCl_2$, 0·002 M; KCl, 0·004 M). A mixture (0·2 ml.) of $K_3Fe(CN)_6$ and $NaHCO_3$ was added from the side arm. Heart-muscle or brain homogenate, 200 mg./vessel. Substrate: 0·05 M-sodium succinate. *A*, heart muscle + sodium succinate; *B*, heart muscle + sodium succinate + heated venom (300 μg./ml.); *C*, brain + sodium succinate; *D*, brain + sodium succinate + heated venom (300 μg./ml.); *E*, corresponding controls without added substrate.

of rat-heart muscle. The heart-muscle preparation, in these experiments, was exposed to the venom for 1 hr. before the substrate was added. Typical results are given in Fig. 5. It is seen that the percentage inactivation due to the heated venom increases with concentration until a limiting value is reached with 150 μg./ml. of venom. Further increase in concentration of venom has apparently little effect on the percentage inhibition. The maximum inhibition produced under these experimental conditions was 55%. The aerobic oxidation of succinate is affected to a greater extent by heated venom than the anaerobic system. This is probably due to the additional inactivation of cytochrome oxidase.

The protective action of succinate on the inhibition of succinic dehydrogenase by heated cobra venom

It was of interest to determine whether succinate would protect succinic dehydrogenase against the inactivating effect of heated venom. The effect of time of contact between the venom and enzyme was therefore studied in two series of experiments, one in which the succinate was present with the tissue and venom throughout the experimental period, and the

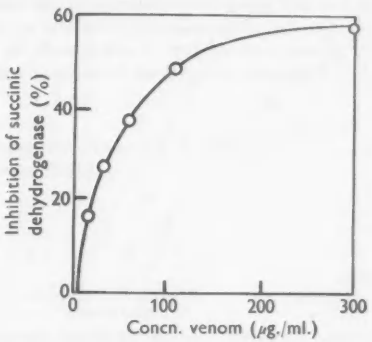

Fig. 5. The effect of different concentrations of heated cobra venom on the percentage inhibition of succinic dehydrogenase activity. Constituents of the medium were the same as those described in Fig. 4. The venom was incubated with 50 mg. rat heart-muscle homogenate and succinate (0·05 M) for 60 min. before the addition of the ferricyanide from the side arm. Percentage inhibitions were determined after an experimental period of 30 min.

other in which the succinate was tipped into a mixture of venom and tissue at various intervals of time.

The results obtained are summarized in Table 2. Comparing the two series of results, it is evident that in absence of succinate the inhibitory effect of venom is greater than in its presence. Thus, succinate protects succinic dehydrogenase against the toxicity of heated venom. Apparently the heated venom attacks the enzyme protein itself and not any of the intermediate co-factors involved in the transfer of hydrogen from the dehydrogenase to the ferricyanide. Binet, Weller & Jaulnes (1937) reported that glutathione added to solutions of crude cobra venom at pH 7·4 or 8·4 considerably reduced its toxicity to animals. The toxicity of purified preparation of neurotoxin of *Naja tripudians* (a variety of cobra) was also reduced by glutathione (Micheel & Schmitz, 1938).

Both in the presence and absence of glutathione (0·01 M), venom inhibits the succinic oxidase system of a rat-brain homogenate by 65 %, after being in contact with the tissue for a total period of 2 hr. The inhibitory factor present in heated venom, therefore, is not inactivated by glutathione under the given experimental conditions.

The effect of heated venom on choline oxidase

Mann & Quastel (1937) showed that in rat liver choline oxidase catalyses the oxidation of choline to betaine aldehyde. As a result of studies on the intermediate carriers involved in this reaction Mann, Woodward & Quastel (1938) concluded that the choline dehydrogenase system consists essentially of the dehydrogenase, cytochrome *c*, cytochrome oxidase and oxygen. It was also shown that this dehydrogenase can reduce ferricyanide under anaerobic conditions. Furthermore, like succinic dehydrogenase, choline oxidase activity is intimately associated with the structure of the cell, and the enzyme has not yet been obtained in a soluble state. In view of the resemblance between the choline- and succinate-oxidizing systems, the action of heated venom on choline oxidase of rat liver was investigated.

Estimations of choline oxidase activity were carried out anaerobically by the manometric method using ferricyanide as the terminal hydrogen acceptor. The homogenate was incubated with the heated venom before the ferricyanide was tipped from the side arm. Typical results are given in Fig. 6.

It will be observed that heated venom causes a pronounced inhibition of choline dehydrogenase. In an experimental period of 50 min. heated venom inactivates 60 % of the enzyme. When crude venom is used (see Fig. 6), the inhibition produced on choline oxidase is much less than that observed in the presence of the heated venom. This observation suggests that crude venom lacks substances, other than those present in the heated venom, capable of destroying choline oxidase, and that some constituents of the crude venom interfere with choline oxidase inactivation by heated venom.

The inactivating action of heated cobra venom on choline oxidase increases with time, the amount of inactivation being similar to that found with succinic dehydrogenase.

The effect of heated venom on anaerobic glycolysis by guinea pig brain homogenate

It has been demonstrated that venom inhibits the aerobic oxidation of glucose. Since anaerobic glycolysis is an important pathway by which glucose may be broken down, it was important to

Table 2. *The protective action of succinate on the inhibition of succinic dehydrogenase by heated cobra venom*

Time interval before tipping $K_3Fe(CN)_6$	Relevant constituents of the system	With succinate present in contact with enzyme and venom: CO_2 evolved in 30 min. (μl.)	With succinate present in contact with enzyme and venom: Percentage inhibition (at 37°)	Succinate tipped with $K_3Fe(CN)_6$: CO_2 evolved in 30 min. (μl.)	Succinate tipped with $K_3Fe(CN)_6$: Percentage inhibition (at 37°)
20 min.	Succinate (0·05 M) + rat-heart muscle (50 mg.)	710	—	750	—
	Succinate (0·05 M) + rat-heart muscle (50 mg.) + 150 μg./ml. heated venom	580	18·0	474	37·0
45 min.	Succinate (0·05 M) + rat-heart muscle (50 mg.)	710	—	770	—
	Succinate (0·05 M) + rat-heart muscle (50 mg.) + 150 μg./ml. heated venom	390	45·0	356	54·5

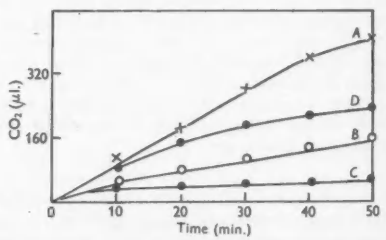

Fig. 6. The effect of heated and crude cobra venom on choline oxidase activity of rat-liver homogenate. Medium: bicarbonate-Locke medium. Mixture of $K_3Fe(CN)_6$ and $NaHCO_3$ (0·2 ml.) added from the side arm. Temperature, 37°. Gas phase, 93 % N_2, 7 % CO_2. *A*, rat liver (100 mg.) + choline chloride (0·01 M); *B*, rat liver (100 mg.) + choline chloride (0·01 M) + heated venom (300 μg./ml.); *C*, rat liver (100 mg.); *D*, rat liver (100 mg.) + choline chloride (0·01 M) + crude venom (300 μg./ml.).

discover whether heated venom can attack the enzyme systems concerned.

Mellanby (1934) observed that, whilst the venoms of *Lachesis atrox*, *L. lanceolatus* and of viper, produced no effect on the anaerobic glycolysis by cancer tissue, cobra venom even in small doses (1 in 1500) inhibited glycolysis. In extension of these studies, Chain (1937) showed that crude venoms of Australian snakes inhibited glycolysis by rabbit-

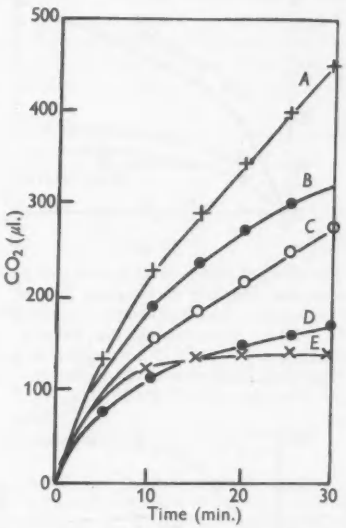

Fig. 7. The effect of heated and crude cobra venom on glycolysis by guinea pig brain homogenate. Medium: K_2HPO_4 buffer (pH 7·4) 0·0025M; $KHCO_3$, 0·025M; $MgCl_2$, 0·006M; Na_4ATP, 0·006M; potassium hexosediphosphate, 0·0025M; sodium pyruvate, 0·005M; DPN, 0·02%; glucose, 0·01M. Enzyme: guinea pig brain homogenate (70 mg./vessel) prepared in 0·08M-nicotinamide and 0·15M-KCl. Temperature, 37°. Gas phase, 93% N_2+7% CO_2. *A*, brain+glucose+heated venom (300 μg./ml.); *B*, brain+glucose; *C*, brain+heated venom (300 μg./ml.); *D*, brain; *E*, brain+glucose+crude cobra venom (300 μg./ml.).

muscle extract and to a lesser extent fermentation. It was reported that the inhibitor in these venoms is thermostable. In the present investigation, the action of heated cobra venom on glycolysis of guinea pig brain homogenate and on brain hexokinase has been examined. The results (Figs. 7 and 8) indicate that the heated venom causes a pronounced activation of both.

Under similar experimental conditions the same quantity of crude unheated cobra venom causes complete inhibition of glycolysis doubtless owing to the presence of DPN-ase and ATP-ase.

The effect of heated venom on the hexokinase activities of brain and yeast

In order to discover whether the activation of glycolysis by heated venom was due to an accelerating effect on hexokinase activity, the action of heated venom on the hexokinase of guinea pig brain homogenate and, also, of an extract of acetone-dried yeast, was investigated. Preliminary experiments had already shown that heat-treated venom

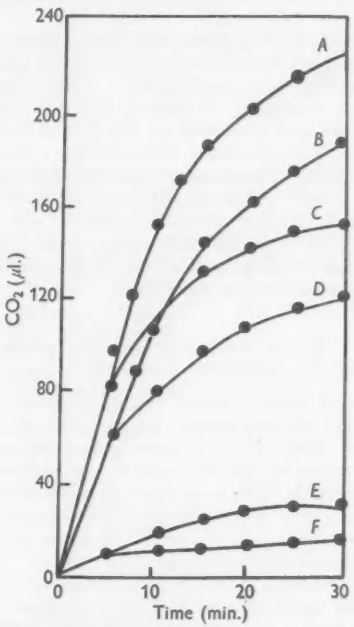

Fig. 8. The effect of heated cobra venom on hexokinase activity of guinea pig brain homogenate. Medium: $KHCO_3$, 0·025M; KF, 0·1M; $MgCl_2$, 0·008M; iodoacetate, 0·005M; glucose, 0·01M; brain homogenate 100 mg./vessel. Temperature, 37°. Gas phase, 93% N_2+7% CO_2. *A*, brain+ATP (0·004M)+heated venom (300 μg./ml.); *B*, brain+ATP (0·004M); *C*, brain+ATP (0·002M)+heated venom (300 μg./ml.); *D*, brain+ATP (0·002M); *E*, brain+heated venom; *F*, brain.

contains no ATP-ase. The results obtained with brain homogenate in the presence of two concentrations of ATP are shown in Fig. 8. It is seen that heated venom causes about 30% increase in hexokinase activity. Results obtained with yeast hexokinase demonstrate that the heated venom has no action on this system.

Although the experimental data are insufficient to determine the nature of the activating factor, it seems likely that the activation of brain glycolysis

by heated venom is connected with its effect on brain hexokinase activity. Furthermore, from the results obtained on the action of venom on glycolysis by brain tissue, it may be concluded that none of the series of enzymes concerned in the anaerobic breakdown of glucose to lactic acid is inactivated by heated venom.

Glucose oxidase ('*notatin*'). This enzyme has flavin-adenine dinucleotide as its prosthetic group (Keilin & Hartree, 1948) and is freely extractable. The enzyme is quite unaffected by the presence of heated cobra venom (Table 4, Exp. 2).

Glucose oxidase of pigeon liver. This enzyme, which also catalyses the oxidation of glucose to gluconic acid, differs from notatin in its requirement of DPN for its activity (Andersson, 1934). A preparation made from acetone-dried pigeon liver was used as the source of enzyme. Heated cobra venom has no inhibitory effect on its activity (Table 4, Exp. 1).

The results obtained with the soluble glucose-oxidizing enzymes from *Penicillium* and from pigeon liver are in contrast to those found with the glucose-oxidizing system of brain which is inhibited by the heated venom (see Fig. 1).

The effects of heated venom on the anaerobic breakdown of pyruvate

As was shown, heated venom produces an inactivation of the enzyme system involved in the aerobic oxidation of pyruvate by brain tissue. As many of the symptoms caused by venom intoxication are believed to result from lesions produced in various parts of the central nervous system, the effect of heated venom on the enzymes concerned with the metabolism of pyruvate, which is known to be involved in the functional activity of the nervous system, was studied in greater detail. Investigations reported here deal with the effect of heated venom on the anaerobic oxidation and dismutation of pyruvate by animal tissues. The results obtained demonstrate that the anaerobic oxidation of pyruvate is very susceptible to the toxic action of heated venom, whilst dismutation is completely unaffected. The anaerobic oxidation of pyruvate by brain tissue was determined by the ferricyanide technique in presence of pyocyanin as a carrier, as described by Michaelis & Quastel (1941).

The results obtained (Fig. 9) demonstrate that heated venom completely inactivates pyruvic oxidase, reducing the evolution of carbon dioxide to the level of the control without substrate. Crude venom was found to produce a similar inhibition. The inhibition produced by heated venom on the anaerobic system increases progressively with time. In this respect it resembles the inhibition produced by heated venom on the aerobic oxidation of pyruvate.

The results of experiments to determine the effect of changes of concentration of venom are shown in Fig. 10. It is evident that the percentage inhibition increases with concentration of the inhibitor until it reaches a maximum of 100% with 75 μg. of venom protein per ml. About 12 μg. of venom protein per ml. are sufficient to inactivate 50% of this enzyme system.

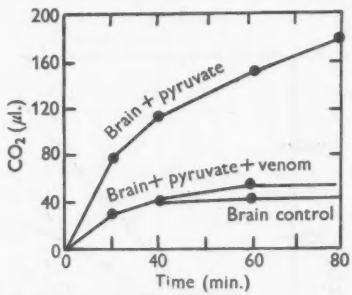

Fig. 9. The effect of heated cobra venom (300 μg./ml.) on the anaerobic oxidation of pyruvate (0·02 M) by rat-brain homogenate. Medium: 0·025 M bicarbonate-Locke medium + 0·3 ml. pyocyanin (3·2 mg./100 ml.). Mixture of $K_3Fe(CN)_6$ and $NaHCO_3$ (0·2 ml.) added from the side arm. Rat-brain homogenate, 125 mg./vessel. Temperature, 37°. Gas phase, 93% N_2 + 7% CO_2.

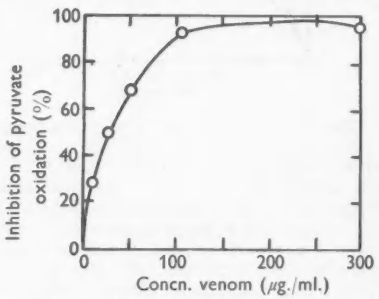

Fig. 10. Effects of variation of concentration of heated venom on the percentage inhibition of the anaerobic oxidation of pyruvate (0·02 M). Heated venom was incubated with the enzyme for 60 min. before the addition of the ferricyanide mixture. The percentage inhibition was calculated after an experimental period of 60 min.

Linkage of toxicity of heated venoms with enzymes associated with cell particulate matter

It is evident from the results already described with succinic, choline and pyruvic dehydrogenases that heated cobra venom inhibits, at rates that increase with time, enzymes whose activities are intimately associated with cell particulate matter. The action of heated venom on a variety of enzyme

systems active in cell-free extracts was therefore studied to discover whether the action of the venom is linked with the physical structure of the enzyme system. In this connexion, a study of the effect of heated venom on 'soluble' pyruvic oxidase was of importance.

Effects of heated venom on enzymes active in cell-free extracts

Pigeon breast. Pigeon-breast muscle has been shown to contain a 'soluble' pyruvic oxidase which catalyses the transfer of hydrogen from pyruvate to molecular oxygen or to ferricyanide under anaerobic conditions (Stumpf *et al.* 1947). This enzyme has now been purified and obtained in a cell-free preparation (Jagannathan & Schweet, 1952).

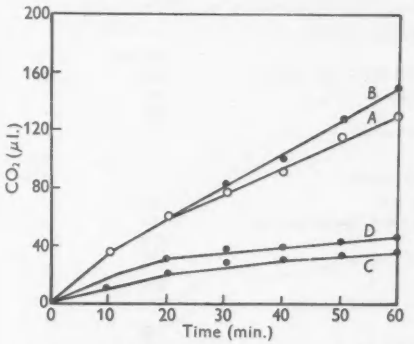

Fig. 11. The effect of heated venom (300 μg./ml.) on the activity of pyruvic oxidase in an extract of pigeon-breast muscle. Medium: 0·025 M-bicarbonate-Locke medium, $K_3Fe(CN)_6 + NaHCO_3$ mixture added from the side arm. Pigeon-breast muscle extract, 0·5 ml./vessel. Temperature, 37°. Gas phase, 93% N_2 + 7% CO_2. *A*, tissue extract + sodium pyruvate (0·02 M); *B*, tissue extract + sodium pyruvate + heated venom; *C*, tissue extract; *D*, tissue extract + heated venom.

The results obtained (Fig. 11) show that heated venom has no effect on the pyruvic oxidase activity of pigeon-breast muscle extract, in marked contrast to its action on the pyruvic oxidase prepared from brain tissue. The completely contrasting susceptibilities shown by these two types of pyruvic oxidase are in agreement with the view that the venom inactivates those enzymes whose activity is dependent on their components having particular spatial arrangement in the cell. These arrangements are presumably maintained by linkages which are susceptible to disruption by the venom.

Pigeon-liver acetone-dried powder. It has been demonstrated that many animal tissues, both in the form of slices (Krebs & Johnson, 1937) and minces (Weil-Malherbe, 1937), can degrade pyruvate under anaerobic conditions by a dismutation reaction in which pyruvate acts both as a donor and acceptor of hydrogen. This reaction is catalysed by enzyme systems which are active in extracts. A study of the effects of heated venom on this system was carried out.

Acetone-dried pigeon liver was used as the source of enzyme. Its activity in dismuting pyruvate was determined manometrically by measuring the CO_2 evolved when sodium pyruvate was tipped into a medium containing the enzyme and sodium bicarbonate (atmosphere 7% CO_2, 93% N_2 at 37°).

The effects obtained with pigeon-liver powder are shown in Table 4, Exp. 6. Heated venom has no inhibitory action on the system of enzymes involved in the dismutation of pyruvate.

Table 3. *The effect of heated venom on the acetylation of sulphanilamide*

Chief constituents of the system (+ sulphanilamide)	Sulphanilamide acetylated (μg.)	Percentage acetylation of sulphanilamide
Pyruvic oxidase system (rat-brain homogenate) + pigeon-liver powder	40·4	50
Pyruvic oxidase system + pigeon-liver powder + 300 μg./ml. heated cobra venom	17·5	22·5
Pyruvic-dismuting system (pigeon-liver powder) + 0·05 M-KF	45·0	60·0
Pyruvic-dismuting system (pigeon-liver powder) + 300 μg./ml. heated cobra venom + 0·05 M-KF	42·0	56·0
Pigeon-liver powder + acetate + ATP (0·003 M) + NaF (0·02 M)	41·0	54·5
Pigeon-liver powder + acetate + 300 μg./ml. heated cobra venom + ATP (0·003 M) + Na (0·02 M)	36·7	46·5

The effects of heated venom have also been studied on a larger variety of enzyme systems, both from animal and other sources, all of which have the common feature of being active in cell-free extracts. It was found (Table 4) that heated cobra venom, in concentrations of 150 μg. venom protein per ml., was without inhibitory action on the following enzyme systems: (1) lactic dehydrogenase of brain and of yeast; (2) malic dehydrogenase of brain; (3) urease—heated venom was found to produce a small inhibitory effect (of the order of 10%) on this enzyme; (4) acetylations of choline and sulphanilamide—recent observations (Braganca & Quastel, 1952) have shown that heated cobra venom exerts no inhibitory effect on the acetylation of choline by extracts of brain in presence of added ATP. The effect of heated cobra venom on the acetylation of

sulphanilamide by extracts of pigeon liver both in presence of added ATP and in presence of metabolizing pyruvate is shown in Table 3. These observations demonstrate that, when energy is supplied in the form of added ATP, the heated venom has only a negligible effect on the acetylation of sulphanilamide. Different results are obtained if the acetylation is made to depend on the respiratory activity of the brain or liver, no ATP being added to the system. If, under these conditions, the energy for the acetylation process is derived from the dismutation of pyruvate, the acetylation of sulphanilamide is not affected by the heated venom; but, when the energy is generated by the oxidation of pyruvate, a pronounced inhibition of the acetylation of sulphanilamide is produced. It is evident from these findings that heated venom affects the acetylation process only indirectly through its effect on the metabolism of pyruvate.

Table 4. *Effects of heated cobra venom on various enzyme systems active in cell-free extracts*

(Details of these experiments in text)

Exp.	Enzyme system	Relevant constituents of the system	CO_2 evolved (μl.)	O_2 uptake (μl.)
1	Glucose oxidase (pigeon liver)	No added substrate	40	—
		Glucose	114	—
		Glucose + heated venom	108	—
2	Glucose oxidase (notatin)	Glucose	—	470
		Glucose + heated venom	—	475
3	Lactic dehydrogenase (rat brain)	No added substrate	88	—
		No added substrate + heated venom	84	—
		Lactate	178	—
		Lactate + heated venom	181	—
4	Lactic dehydrogenase (yeast)	Sodium lactate	920	—
		Sodium lactate + heated venom	895	—
5	Malic dehydrogenase (rat brain)	Malate	108	—
		Malate + heated venom	103	—
		No added substrate	50	—
		No added substrate + heated venom	50	—
6	Pyruvic dismutase (pigeon liver)	Pyruvate	187	—
		Pyruvate + heated venom	178	—
7	Pyruvic oxidase (pigeon-breast muscle)	No added substrate	50	—
		No added substrate + heated venom	45	—
		Pyruvate	180	—
		Pyruvate + heated venom	170	—
8	Urease (400 μg.)	Urea	380	—
		Urea + heated venom	340	—

The nature of the toxic factor in heated cobra venom

The progressive increase in inhibitory activity of heated venom with time suggests that the inhibitor may be an enzyme degrading certain essential components of cellular structure. This idea is supported by the fact that further heating in alkaline solution (pH 10·0) destroys its activity. Most enzymes present in venom are not active in the crude heated product but lecithinase A has been reported to be remarkably resistant to heat (Hughes, 1935). Since the interaction between lecithinase A of cobra venom and lecithin leads to the liberation of free acid groups, it is possible to obtain a measure of lecithinase activity using the Warburg manometric technique (Zamecnik, Brewster & Lipmann, 1947).

The lecithinase activities of crude and heated cobra venom (Haffkine) were determined using a purified sample of lecithin. The results obtained using 1 mg. of venom/vessel and varying concentrations of the lecithin are given in Figs. 12*a* and *b*. These demonstrate that with heated venom, the limiting velocity of breakdown of lecithin is reached with 40 mg. lecithin/vessel.

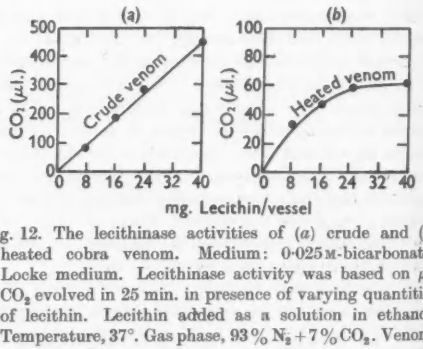

Fig. 12. The lecithinase activities of (*a*) crude and (*b*) heated cobra venom. Medium: 0·025 M-bicarbonate-Locke medium. Lecithinase activity was based on μl. CO_2 evolved in 25 min. in presence of varying quantities of lecithin. Lecithin added as a solution in ethanol. Temperature, 37°. Gas phase, 93 % N_2 + 7 % CO_2. Venom, 1 mg./vessel.

The action of venom phospholipases on heated brain and heart tissues

Results shown in Fig. 13 indicate the effects obtained when crude and heated venoms react with brain-tissue homogenate, previously heated for 30 min. at 100°. It is evident from these observations that crude and heated venoms liberate acid groups from constituents of the brain, indicating a probable breakdown of the brain phospholipins. The course of the reaction seems to be similar to that observed with lecithin. Though crude venom is more active than the heated product, it is evident that the latter is capable of bringing about the disruption of structures in which phospholipins are concerned in the brain. It is reasonable to consider that this may be the mechanism by which the heated venom

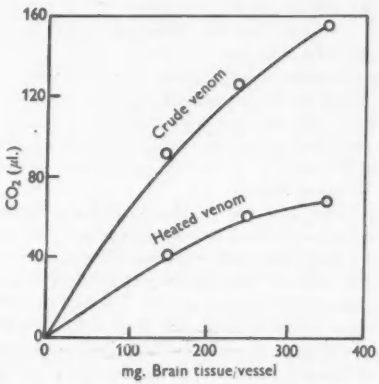

Fig. 13. The effects of phospholipases of crude and heated cobra venoms on heated brain tissue. Medium: 0·025 M-bicarbonate-Locke medium. Experimental period, 30 min. Temperature, 37°. Gas phase, 93% N_2 + 7% CO_2. Venom, 1 mg./vessel.

inactivates a number of the enzyme systems essential for the respiratory processes. Such inactivation may also be sufficient to explain many of the toxic effects resulting from venom administration.

Russell's viper venom, which lacks the neurotoxic effects of cobra venoms, shows a much smaller lecithinase activity. Several experiments have demonstrated that its activity (after heating) is less than one-third of that of heated cobra venom.

Heated Russell's viper venom produces a smaller inhibition of succinate oxidation than that given by the heated cobra venom. The results obtained on the effects of heated Russell's viper venom and cobra venom on the overall oxidation of succinate are given in Fig. 14. This finding lends further support to the view that the inhibitory action of the heated venom on the complete oxidation of succinate is associated with the lecithinase activity of the venom.

The effects of calcium ions on the inhibitory action of heated venom on respiratory enzyme systems

Delezenne & Ledebt (1911*a*, *b*) investigated the properties of lecithinase in venoms responsible for haemolysis and showed that Ca^{++} had marked effects on this phenomenon. The activating effect of calcium on haemolysis by cobra venom was later confirmed by Holden (1934).

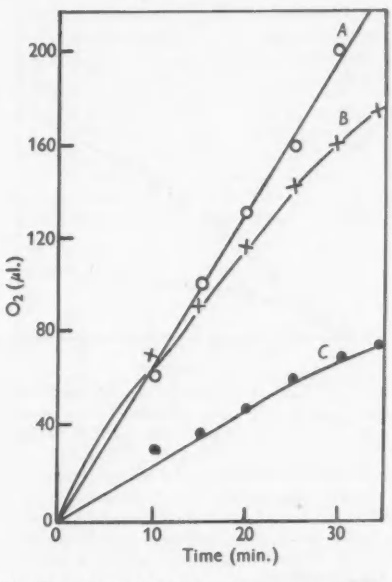

Fig. 14. The effect of heated cobra venom and heated Russell's viper venom on the oxidation of succinate by rat-brain homogenate. Medium: 0·02 M-phosphate-Locke. Temperature, 37°. Gas phase, air. Homogenate, 150 mg./vessel. *A*, brain + sodium succinate (0·05 M); *B*, brain + sodium succinate (0·05 M) + heated Russell's viper venom (300 μg./ml.); *C*, brain + sodium succinate (0·05 M) + heated cobra venom (300 μg./ml.).

The activating action of calcium ions on the toxic effect of heated cobra venom was shown with the system of enzymes catalysing the aerobic oxidation of glucose. The results obtained are shown in Fig. 15. It is evident that a sharp increase in the inhibitory action of the heated venom is brought about by the greater concentration of Ca^{++}. Furthermore, the initial lag period preceding the onset of the inhibitory effect is no longer detectable.

DISCUSSION

It is evident from the observations of the action of heated venom on a variety of enzyme systems that the inhibitor present is not a general enzyme poison. Heated venom has been found to attack the following enzyme systems to varying extents: (1) Systems contained in brain homogenate concerned with the oxidation of glucose, pyruvate, glutamate, succinate, α-ketoglutarate, and fructose. (2) Pyruvic dehydrogenase of rat brain. (3) Succinic dehydrogenase of brain and heart-muscle tissues. (4) Cytochrome oxidase of brain tissue. (5) Choline oxidase of rat liver.

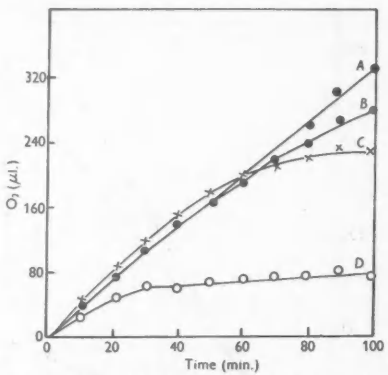

Fig. 15. The effect of calcium on the inhibitory action of heated venom on glucose oxidation by rat-brain homogenate. Medium: Na_2HPO_4 buffer (pH 7·4) 0·02 M; NaCl, 0·13 M; KCl, 0·004 M. Venom, 300 μg./ml. Glucose, 0·02 M. Rat-brain homogenate, 150 mg./vessel. Temperature, 37°. Gas phase, air. *A*, brain + glucose + $CaCl_2$ (0·036 M); *B*, brain homogenate + glucose + $CaCl_2$ (0·002 M); *C*, brain homogenate + glucose + heated venom + $CaCl_2$ (0·002 M); *D*, brain homogenate + glucose + heated venom + $CaCl_2$ (0·036 M).

The effects produced on the overall oxidation of various substrates by brain tissue are likely to be due to the inactivation of the pyruvic oxidase and cytochrome oxidase systems.

Closer examination of the results shows that the numerous enzyme systems not attacked by the heated venom have one common feature; they are all 'soluble' and active in aqueous tissue extracts, and their activities seem to be independent of cellular structure. Those enzymes which are inactivated by heated venom are associated with mitochondrial bodies and apparently require specific structural configuration for their activities. The succinic oxidase system is a typical example. Keilin & Hartree (1949) distinguish between two types of factors affecting this system; those reacting specifically with a catalyst in the system and those affecting the colloidal structure of the enzyme components. Keilin & Hartree (1940) conclude that the activity of succinic oxidase is intimately associated with the integrity of the tissue particles, stating that 'efficiency of the system depends, however, not only on the integrity of its components, but also on that of the colloidal structure which supports them and assures their mutual accessibility'. Swanson & Artom (1950) have shown that a large amount of phospholipin material, present in firm combination with protein, is associated with the mitochondria. These findings, taken in conjunction with the reports that the succinic oxidase system (Hogeboom, Schneider & Pallade, 1948), pyruvic oxidase (Kennedy & Lehninger, 1948), and choline oxidase (Kensler & Langemann, 1951) are located in the mitochondria, suggest that heated venom attacks the lipoprotein complex which holds together in a particular spatial configuration the different components of these enzyme systems.

The presence of lecithinase in heated venom has been directly demonstrated by the manometric technique. By this method, it has been possible to show that heated venom can degrade, enzymically, certain structures, probably containing phospholipin, in brain tissue.

The large activity of the inhibitory factor in heated venom, which in concentrations containing 10 μg. protein/ml. can bring about 50 % inactivation of brain pyruvic oxidase, together with the facts concerning the kinetics of the inhibition, furnish additional evidence in favour of an enzymic process being involved in the inactivation of enzyme systems by the heated venom. The accelerating action of Ca^{++} on the inactivation of glucose oxidation of brain by heated venom, coupled with the marked activating action of Ca^{++} on the lecithinase activity of venom, provides a direct link between lecithinase activity and the inhibitory effects of the venom on the various enzyme systems.

Heated venom inhibits specifically the pyruvic oxidase associated with cell structure and has no effect on pyruvic oxidase that can be extracted in cell-free aqueous solution from pigeon-breast muscle. Clearly, then, heated venom may be of direct service as a biochemical tool in its ability to discriminate between enzymes that have similar biochemical properties, but which differ from each other in their physical structure.

The observations reported here suggest that heated snake venom exerts its inhibitory effects on tissue enzyme systems by degrading, through the action of a phospholipase or phosphotidase, mitochondrial structures upon whose integrity the enzyme systems concerned depend for their optimal activities. It seems possible that venom lecithinase, by its pronounced inhibitory action on tissue

respiratory systems, as well as by its effect on acetylations (Braganca & Quastel, 1952), is concerned with some of the neurological disturbances that characterize the toxic action of snake venoms in the animal.

SUMMARY

1. Investigations have been made on the effects of snake venoms heated at 100° for 15 min. on a variety of enzyme systems. Such venoms still retain toxicity to animals.

2. The enzymic systems concerned with the oxidation of glucose, pyruvate, L-glutamate, succinate, α-ketoglutarate and fructose by brain homogenates are inhibited by small quantities of the heated venoms.

3. Pyruvic dehydrogenase of brain, succinic dehydrogenase of brain and heart, cytochrome oxidase of brain, and choline oxidase of liver are all vigorously attacked.

4. The following systems are not attacked: anaerobic glycolysis by rat brain, glucose fermentation by yeast extract, pyruvic acid dismutation by pigeon-liver, hexokinase, glucose oxidase of liver, and glucose oxidase of fungi (notatin), soluble pyruvic dehydrogenase of pigeon-breast muscle, lactic and malic dehydrogenases, and urease. Cytochrome *c* is not broken down.

5. The enzyme systems that are not attacked by heated venom are distinguished from those that are attacked by being active in aqueous cell-free tissue extracts and are apparently independent of cellular structures for their activities.

6. Heated snake venom contains a heat-stable enzyme (probably a lecithinase) that attacks brain phospholipins or lipoproteins. Its inhibiting effect on a susceptible enzyme system is enhanced by calcium ions which activate lecithinase.

7. It is suggested that heated venoms exert their inhibitive effects by enzymic degradation of mitochondrial structures upon whose integrity or particular spatial configurations the enzyme systems concerned depend for their optimal activities.

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The Phytase of Wheat

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Phytase catalyses the hydrolysis of phytic acid (inositol hexaphosphoric acid) to inositol and free orthophosphate. It was one of the first enzymes which liberate inorganic phosphate from organic phosphorus compounds to be described (Suzuki, Yoshimura & Takaishi, 1907), and it has a wide distribution in plant and animal tissues, in many species of fungi and in certain bacteria. It has not, however, been so intensively studied as many of the other phosphatases, although in recent years Courtois and his collaborators have carried out extensive work on its occurrence, specificity and mechanism of action (cf. Fleury & Courtois, 1937, 1947; Courtois, 1945, 1947; Courtois & Biget, 1943; Courtois & Joseph, 1947; Courtois & Plumel, 1947; Courtois & Perez, 1948).

In cereal grains a considerable proportion of the total phosphorus is present as phytate; in wheat, for example, the phytin P constitutes 70–75 % of the total P of the grain (Booth, Carter, Jones & Moran, 1941). This is probably the source of the suggestion, frequently made, that a principal function of phytase in grains is to provide inorganic phosphate from phytate in the initial stages of germination. In this connexion Bernardini & Morelli (1912), Albaum & Umbreit (1943), de Turk, Holbert & Howk (1933) and Giri & Sreenivasan (1938) have shown that the proportion of inorganic phosphate to phytate increases progressively during germination with wheat, oats, maize and rice respectively. It has also been observed that the phytase activity of grains increases on germination, in barley by Luers & Malsch (1929), in wheat by Bernardini & Morelli (1912) and in oats by Albaum & Umbreit (1943). However, Essery (1951), although observing an increased phytase activity when barley grains germinate, found that the concomitant decrease of phytate P is relatively small and he doubts the validity of the simplified conception of phytase function mentioned above.

The relatively high content of phytate in cereals is a factor of importance in human and animal nutrition, particularly where whole grain or high-extraction products are concerned (the ratio of phytate P to total P increases markedly in the outer layers of the grain). This is because phytate can interfere with the assimilation of calcium and iron, and possibly other metal ions, due to these metals forming phytates which are highly insoluble over a wide pH range. McCance & Widdowson (1935) observed that human subjects on a high-phytate, low-calcium diet develop a negative calcium balance, and this observation provided the basis for the present practice of adding calcium carbonate to high-extraction flours. If phytate is hydrolysed by phytase then the immobilization of calcium will be decreased, and the interesting observation of Walker, Irving & Fox (1948), that in some subjects on high-phytate, low-calcium diets there is a gradual adaptation so that calcium balance is eventually restored, may perhaps be explained by an increased phytase activity of the intestinal flora.

It is useful, for both nutritional and practical reasons, to know how phytase is distributed among the different anatomical parts of the wheat grain and on its thermal stability in wheat and wheat products. No detailed work on these questions appears to have been published, and they have therefore been examined as part of a general study of wheat phytase which is described in this paper.

EXPERIMENTAL

Materials

Sodium phytate. Two products have been obtained from Ciba Ltd., 'phytin' and 'sodium phytinate'. The phytin (mixed calcium and magnesium salt of phytic acid), gives, on analysis, the correct phosphorus content and the inorganic phosphate content is about 1 % of the total P. If phytin is used as the enzyme substrate difficulties arise from the

separation of calcium phosphate from solution as the reaction proceeds, and the incomplete solubility of phytin at pH values greater than 5·0.

The 'sodium phytinate' supplied by Ciba Ltd. contained about 25% of its total P as free orthophosphate and was thus far from pure. Posternak (1919), Harrison & Mellanby (1939) and Courtois (1948) have indicated that sodium phytate ($C_6H_6O_{24}P_6Na_{12}.35H_2O$) may readily be crystallized. Many attempts to crystallize this salt in these laboratories have been unsuccessful and this has also been the experience of Essery (private communication).

Sodium phytate solutions, prepared as follows from commercial phytin, have therefore been used as the substrate: About 10 g. of phytin are dissolved in 50 ml. of water with the aid of the minimum amount of concentrated HCl. 20% (w/v) $FeCl_3$ in 2N-HCl is added until the supernatant obtained on centrifuging gives no further precipitate with the reagent. The white precipitate of ferric phytate is washed by centrifugation with three 50 ml. portions of N-HCl and five of water. The ferric phytate is then evenly suspended in water and 10N-NaOH added with vigorous stirring until, on centrifuging, a water-clear, colourless supernatant is obtained. This supernatant is decanted, filtered, the pH adjusted to 5·15 with HCl and the volume made up to 200 ml. before determination of inorganic and total P. Usually the orthophosphate content is less than 0·01% of the total P and the yield is about 70%, although the latter varies somewhat, probably due to the degree of fineness of suspension of the ferric phytate prior to double decomposition.

The work of Hanes & Isherwood (1949) on the separation of phosphate esters on paper chromatograms, suggested a possible means of testing the purity of sodium phytate solutions prepared in this way. Using *tert.*-butanol-picric acid with 24 hr. downward runs on Whatman no. 1 filter paper, the preparations showed only one phosphorus-containing spot, but a solution of Ciba 'sodium phytinate' showed three such spots; one due to phytate, one to orthophosphate and a third, having an intermediate R_F value, due to unidentified material.

Using the procedure of Hanes & Isherwood (1949) attempts were also made to separate the possible intermediates of phytase action, i.e. the various inositol phosphates. Using a wide range of solvent systems, it was found possible to demonstrate the accumulation of free organic phosphate by paper chromatography, but not the existence of intermediate inositol phosphates.

Preparation of partially purified phytase. 30 g. of a wholemeal from Manitoba wheat are extracted for 6 hr. at 0–4° with 175 ml. distilled water (pH of the suspension is about 6·0). The suspension is centrifuged to remove the bulk of the solid matter and the supernatant filtered to give 150 ml. of a clear solution pH 5·9. This is poured with vigorous stirring into 600 ml. ice-cold acetone, allowed to stand 10 min. and the bulk of the supernatant liquor decanted. The precipitate is collected on a Büchner funnel and washed with acetone, acetone-ether (1:1) and finally ether: about 1·4 g. of a white powder are obtained. This is extracted for 2 hr. at 0–4° with 75 ml. distilled water and then filtered. The solution is saturated with $(NH_4)_2SO_4$, kept for 2 hr. at 0–4° and the precipitate which forms is collected on a Buchner funnel and dried *in vacuo* over anhydrous $CaCl_2$.

The dry material (0·4 g.) is dissolved in 20 ml. distilled water and dialysed 48 hr. at 0–4° against three changes of 2 l. distilled water. A slight precipitate which forms during dialysis is filtered off. The phytase activity of the final solution recovered on a dry-weight basis is usually about 20 times that of the original wholemeal with a recovery of about 25%.

Methods

Phosphate estimation. The methods used for the determination of total P and orthophosphate P were those described by Allen (1940): the colour densities were read on a Spekker instrument against a reagent blank using spectrum red (H. 508) and heat-resistant filters. The amidol bisulphite reagent used was made up fresh daily and filtered before using.

It was noticed, in the estimation of orthophosphate P in the presence of phytate P, that the $HClO_4$ appeared to hydrolyse phytate relatively rapidly at room temperature. Experiments showed that phytate and pyrophosphate were hydrolysed by the $HClO_4$ at room temperature liberating free orthophosphate, but that α-glycerophosphate and phenyl phosphate were resistant to hydrolysis in this way. The rate of hydrolysis of phytate by $HClO_4$ increases rapidly with temperature but is zero at 4°. Following these observations, since most of the estimations were of orthophosphate in the presence of phytate, solutions in which orthophosphate was to be determined were read on the Spekker between 5 and 10 min. after mixing with the reagents and making to volume.

In the course of inhibitor studies on phytase it was noted that the hydrolysis of phytate by $HClO_4$ appears to be catalysed by tellurite and selenite ions.

Method of following enzyme digestions. Material containing the enzyme, either a wholemeal or a purified preparation, equivalent to 200–1000 'phytase units' (in this paper one phytase unit is the amount of enzyme releasing 1 μg. of orthophosphate P from $1·6 \times 10^{-3}$M-phytate at pH 5·15 and 55° in 1 hr.) is weighed or pipetted into a 50 ml. Erlenmeyer flask. To this is added 5 ml. of 0·2M-acetate buffer, pH 5·15, containing 0·004M-$MgSO_4$, prewarmed to 55°, followed by the appropriate amount of phytate solution at 55° to bring the total volume to 10 ml. and the final substrate concentration to approximately 300 μg./ml. phytate P (i.e. about $1·6 \times 10^{-3}$M). A 2 ml. sample of the suspension or solution is removed immediately and the remainder incubated at 55°, further 2 ml. samples being removed after appropriate time intervals.

The 2 ml. samples are added to 1 ml. 10% (w/v) trichloroacetic acid, filtered and portions of the clear filtrates are used for the determination of orthophosphate P.

RESULTS

Properties of phytase in wholemeal and in a partially purified state

Progress curves. Using 2 g. samples of Manitoba wholemeal in one case and 1·25 ml. samples of a purified preparation (1925 units/ml.) in the other, progress curves of the hydrolysis of phytate have been plotted (Fig. 1). In both experiments the final volume was 25 ml. in standard buffer; the phytate concentration was $1·35 \times 10^{-3}$M in the wholemeal experiment and $1·6 \times 10^{-3}$M in the purified enzyme

experiment. The liberation of orthophosphate P was followed as described under Methods.

pH *Curves.* Samples of Manitoba wholemeal (500 mg.) and 1 ml. samples of a purified preparation diluted to contain 1000 units/ml. have been used to establish the pH-activity relationship at 55°. Buffers of 0·1 M-acetate over the range pH 3·8–5·8 were used to maintain the pH. The results, as shown in Fig. 2, agree very closely for both experimental series and show an optimum pH of 5·15 with a rapid diminution in activity on either side of this optimum. Various figures ranging from 5·0 to 5·5 have been reported in the literature as the optimum pH of phytase: for example, Luers & Silbereisen (1927) quote 5·2 for the optimum pH of malt phytase measured at 48°.

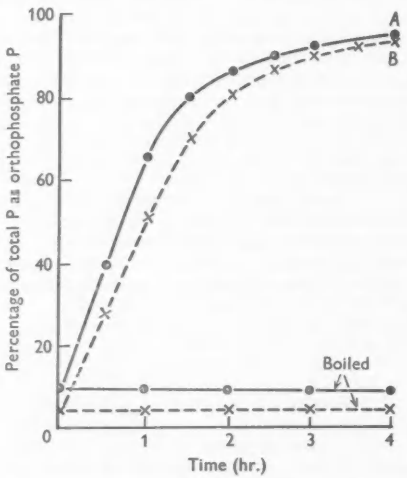

Fig. 1. Progress curves for phytase at 55°. Curve *A*, wholemeal (2 g. in 25 ml. buffered substrate, pH 5·15); curve *B* purified phytase preparation (1925 units in 25 ml. buffered substrate, pH 5·15). The boiled controls are also shown, the wholemeal being suspended in 10 ml. of water and boiled for 5 min.

Effect of enzyme concentration. Varying amounts, up to 750 mg. of the Manitoba wholemeal, and up to 3 ml. of a purified preparation (1925 units/ml.) were assayed for activity under the usual conditions. The results, which are shown in Fig. 3, show linearity of response up to 50 mg. wholemeal/ml. digest and 400 units purified enzyme/ml. digest: the latter corresponds to rather less than twice the enzyme concentration of the former. Luers & Silbereisen (1927) obtained a corresponding value of 200 units/ml. digest for the limit of linearity of response with malt phytase.

Effect of substrate concentration. Using 250 mg. samples of wholemeal and 1 ml. samples of a purified

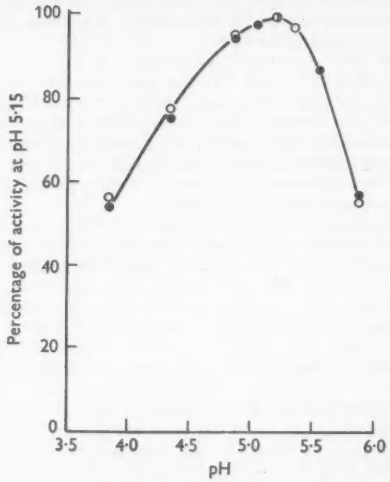

Fig. 2. Influence of pH on phytase activity. Initial reaction velocities (expressed as percentages of the maximum activity) measured at 55° in 0·1 M-acetate buffers in the presence of 0·002 M-$MgSO_4$ and $1{\cdot}61 \times 10^{-3}$ M-phytate; using wholemeal (●), and a purified preparation (○).

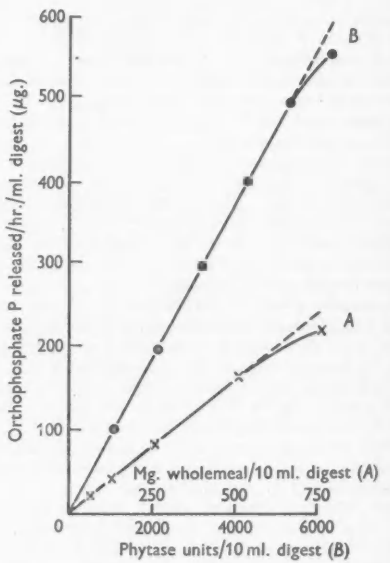

Fig. 3. Effect of enzyme concentration at 55°. Initial reaction velocities measured in buffered substrate, pH 5·15; wholemeal (*A*), purified phytase preparation (*B*).

phytase preparation (1000 units/ml.), initial reaction velocities have been measured at initial phytase concentrations varying from $0{\cdot}27 \times 10^{-3}$ to $1{\cdot}74 \times 10^{-3}$ M with the wholemeal, and from $0{\cdot}79 \times 10^{-3}$ to $3{\cdot}58 \times 10^{-3}$ M with the purified phytase preparation. Reciprocal plots made as described by Lineweaver & Burk (1934) give straight lines and from the intercepts on the arcs the Michaelis constant (K_m) and the maximum reaction velocity (V) have been calculated and are given in Table 1.

Table 1. *Effect of substrate concentration on phytase activity*

(The following figures have been calculated from graphs of $1/v$ against $1/s$: the initial reaction velocities (I.R.V.) at various substrate concentrations being measured under the standard conditions.)

	Michaelis constant (K_m)	Maximum I.R.V. (V) (μg. P/hr./ml. digest)
Wholemeal exp.	$0{\cdot}29 \times 10^{-3}$ M	127
Purified enzyme exp.	$0{\cdot}33 \times 10^{-3}$ M	127

Effect of temperature of incubation. Samples of Manitoba wholemeal (500 mg.) were used, in 20 ml. of buffer-substrate-$MgSO_4$, over a range of incubation temperatures. The progress curves at the different temperatures are plotted in Fig. 4 and these show the optimum temperature of activity to be about 55°. Luers & Silbereisen (1927) obtained a value of 48° for the optimum temperature of malt phytase, while Collatz & Bailey (1921) and Kolobkowa (1936) found 55° as optimum for wheat phytase.

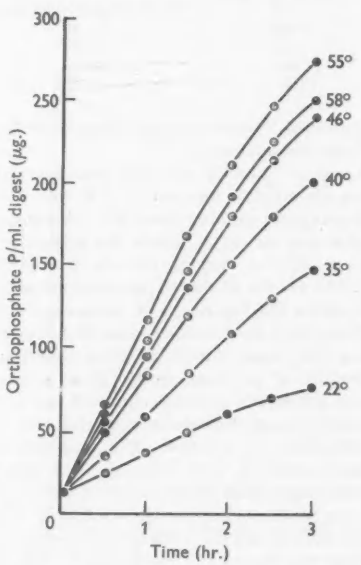

Fig. 4. Effect of temperature on the progress curve of phytase in wholemeal. Measured in buffered substrate, pH 5·15.

Later experiments on the thermal inactivation of phytase showed that some inactivation of the enzyme occurs at 55° in the absence of the substrate and hydrolysis products. It is not known to what extent these may protect the enzyme from heat inactivation. The temperature coefficients $\left(\frac{q_{t+10°}}{q_t}\right)$ have been calculated from the data given in Fig. 4 and are given in Table 2.

Table 2. *Temperature coefficients of activation for wholemeal phytase*

(Derived from the data constituting Fig. 4.)

Temperature interval	$\frac{q_{t+10°}}{q_t}$
25–30°	1·64
35–45°	1·74
45–55°	1·20

Table 3. *Activation of a dialysed phytase preparation by* Mg^{++} *ions*

(Initial reaction velocities measured in 0·1 M-acetate, pH 5·15 and $1{\cdot}58 \times 10^{-3}$ M-phytate at 55°.)

Molarity of Mg^{++} ions	I.R.V. (μg. P/hr./10 ml. digest)
0·0	108
0·0002	188
0·0005	228
0·001	246
0·002	254
0·005	253
0·01	254

Activation by magnesium ions. A phytase preparation consisting of an exhaustively dialysed, filtered, aqueous extract of the Manitoba wholemeal was assayed at different concentrations of $MgSO_4$, other conditions being as described in the Methods section. Before dialysis this preparation was assayed at 253 units/ml. The results, given in Table 3, show that some fall in activity occurs on prolonged dialysis, which may be restored by the addition of Mg^{++} ions at concentrations of 0·002 M and greater. The reduction in activity, however, is not complete and phytase differs in this respect from certain other phosphatases which are completely inactive in the absence of Mg^{++} ions.

Effect of various salts on phytase activity. Using a filtered 3 hr. aqueous extract of wholemeal as the enzyme preparation (275 units/ml.) a number of inorganic and organic salts were tested for inhibitory or stimulatory properties at a final concentration of 0·01 M. The enzyme digestions were

carried out as usual except that no $MgSO_4$ was added. The results are recorded in Table 4, and it is seen that sodium azide, alkaline oxalates and calcium chloride apparently activate the enzyme: the $CaCl_2$ probably does so by disturbing the equilibrium through removal of calcium phosphate from solution. Sodium citrate also appears to activate the enzyme although the response is somewhat irregular.

Sodium fluoride, potassium cyanide and yeast extract, final concentration 0·5% (w/v), partially inhibit the enzyme whereas zinc and manganese sulphates completely inhibit. Zinc and manganese ions probably act by removing the phytate ions from solution since they both cause precipitation in the mixtures.

Table 4. *The action of various salts on the phytase activity of a filtered, aqueous extract of wholemeal*

(Activities are measured at 55° in 0·1M-acetate, pH 5·15, and $1·58 \times 10^{-3}$M-phytate. The salts, are added to a final concentration of 0·01M; the yeast extract to 0·5% (w/v).)

Substance added	Activity (μg. P/hr./ml. enzyme)	Percentage of control
None	273, 275, 274	—
Sodium lactate	273	100
Sodium sulphate	274	100
Magnesium sulphate	273	100
Sodium fluoride	138	50
Potassium cyanide	192	71
Yeast extract (Difco)	190	70
Sodium citrate	430, 303, 357	156, 111, 130
Sodium oxalate	340	124
Ammonium oxalate	342	125
Sodium azide	310, 312	113
Calcium chloride*	364, 360	132
Zinc sulphate*	0	0
Manganese sulphate*	0	0

* These three salts all caused precipitation in the digestion mixtures.

Sodium lactate, sodium sulphate and magnesium sulphate have no effect on the activity of the enzyme under these conditions.

Reproducibility of the assay of phytase in wholemeal. Manitoba wholemeal (250 mg., 88·2% dry matter) was used for the determination of phytase activity, under the standard conditions, on seven different occasions over a period of 3 weeks. The values obtained for the initial reaction velocity expressed as μg. inorganic P released/hr./mg. dry weight were: 5·26, 5·13, 5·21, 5·29, 5·19, 5·24, 5·25; giving a mean value of 5·23 with a standard deviation (σ) of 0·053.

Thermal inactivation of phytase

Inactivation in steam-treated whole wheat grains. Samples of Manitoba wheat (500 g.) were heated to various temperatures by live steam, the wheat being thoroughly shaken in the stream of steam. The temperature was then maintained by feathering the steam supply. After a total time in the steamer of 6 min. the wheat was rapidly cooled and dried back to 500 g. weight at 30° before milling to 100% extraction. Samples of the resulting wholemeals (500 mg.) were assayed for phytase activity and the results are shown in Table 5. The moisture content of the wheat increased from 12% to about 18% during the time in the steamer.

Inactivation in wholemeal. As the rate of thermal inactivation of an enzyme may vary with the moisture content of the medium and since, in the preceding experiment, the moisture content was not controlled, the following experiment was carried out with wholemeal (88·2% dry matter) from Manitoba wheat.

Samples (1 g.) of wholemeal were sealed into thin-walled glass ampoules which were immersed in a water bath maintained at various temperatures up to 100° for exactly 10 min. The ampoules were then rapidly cooled, opened and the phytase activity of the wholemeal determined.

Table 5. *Inactivation of phytase in whole wheat by steaming*

(The activities of wheat treated with steam to maintain various temperatures for 6 min. prior to milling to a wholemeal, determined at 55° in 0·1M-acetate, pH 5·15, 0·002M-$MgSO_4$ and $1·61 \times 10^{-3}$M-phytate.)

Temperature of treatment (°)	Activity (% of untreated control)
62·8	98·5
73·8	77·0
82·2	17·0
100	12·6

The results (Fig. 5) indicate that inactivation becomes measurable, for a period of 10 min. heating, at temperatures greater than 80°. A similar experiment was set up in which the ampoules were heated at 81° for varying periods of time up to 1·5 hr. The results of this experiment are shown in Fig. 6 where the logarithm of percentage activity remaining is plotted against time of heating. The curve is not linear, which indicates that the heat inactivation of phytase, under these conditions, does not follow the unimolecular rate law which is frequently observed with other enzymes.

Inactivation in solution. The inactivation of phytase in solution (705 units/ml.) has been similarly investigated at temperatures from 40 to 70°. From the results, which are shown in Fig. 7, it was decided that 55 and 65° were suitable temperatures for observing the rate of inactivation. The results of these experiments are shown in Fig. 8 where deviation from the unimolecular expression for heat inactivation is again apparent.

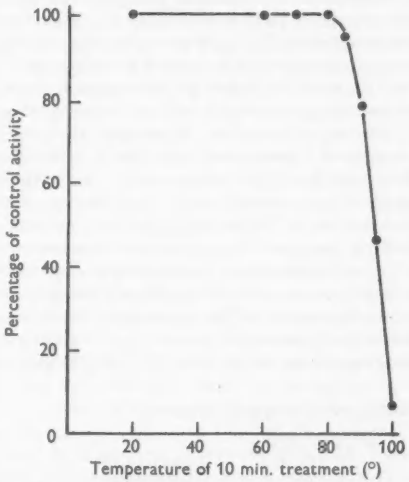

Fig. 5. Thermal inactivation of phytase in wholemeal. Activities of samples of wholemeal heated to various temperatures in sealed ampoules for 10 min., determined in buffered substrate, pH 5·15, at 55°.

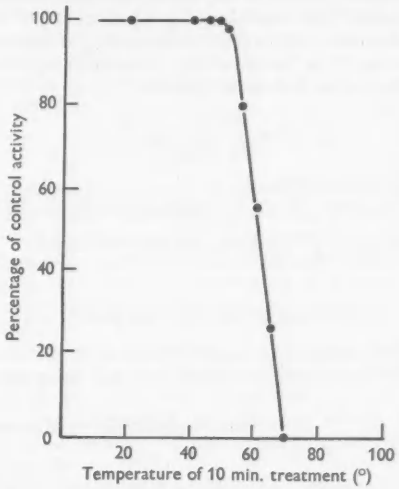

Fig. 7. Thermal inactivation of phytase in solution (705 units/ml.). Activities of samples of a partially purified solution of phytase heated to various temperatures in sealed ampoules for 10 min., determined in buffered substrate, pH 5·15, at 55°.

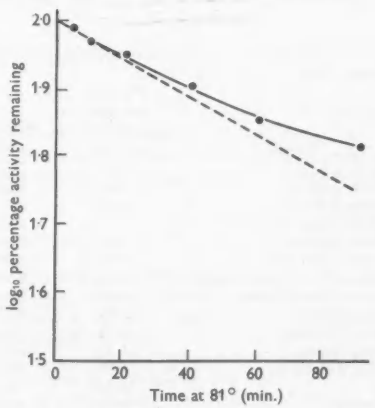

Fig. 6. Thermal inactivation of phytase in wholemeal. Activities of samples of wholemeal heated to 81° in sealed ampoules for various times, determined in buffered substrate, pH 5·15, at 55°.

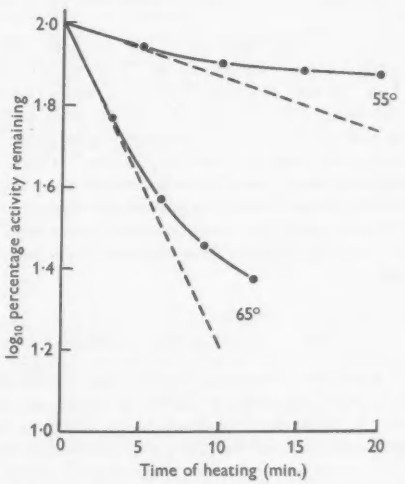

Fig. 8. Thermal inactivation of phytase in solution (705 units/ml.). Activities of samples of a phytase preparation heated to 55 and 65° for varying periods of time determined in buffered substrate, pH 5·15, at 55°.

By constructing the tangents to the two curves obtained in Fig. 8 we can derive the 'pseudomonomolecular' rate constants: $k_{55°} = 0{\cdot}028$, $k_{65°} = 0{\cdot}180$. An indication of the order of the energy of inactivation can then be obtained by substituting these values in the Arrhenius equation:

$$E = \frac{RT_1T_2}{(T_2 - T_1)} \log_e \frac{k_2}{k_1},$$

whence $E = 41\,600$ cal.

The value of the temperature coefficient of inactivation $\frac{k_{t+10}}{k_t}$ for the interval 55–65° is 6·5.

Distribution of phytase in the wheat grain

The various anatomical fractions of the wheat grain have been dissected out and assayed for phytase activity. The results for the soft wheat, Cappelle Deprez, are shown in Table 6, and they provide direct evidence for the suggestion of Widdowson (1941), made on the basis of baking tests, that phytase is more dispersed throughout the wheat grain than its substrate, phytin. It has also been found that an increase of 6·5-fold in the phytase activity occurs on germination of this wheat.

Typical values are non-germinated activity 3·17, and 5-day-germinated 20·6 phytase units/mg. dry weight.

Table 6. *Distribution of phytase in the wheat grain (Cappelle Deprez)*

Fraction	% by wt. of whole grain	Phytase activity (μg. P/hr./mg. dry wt.)	Percentage of total phytase
Wholemeal	100	3·41	100
Endosperm	82·5	1·29	34·1
Germ	1·0	9·07	2·9
Scutellum	1·5	31·8	15·3
Epidermal layers	4·5	1·32	1·9
Testa + cross-layer	3·5	4·34*	4·8
Aleurone	7·0	17·7	39·5

* This figure is obtained by difference from the figures obtained for the epidermal layers and the total pericarp.

Phytase content of various wheats

A number of different wheats, all harvested in 1951, have been examined for phytase activity. Small samples were ground to wholemeals in a mechanical mill and the phytase activities measured by the standard procedure. The results, shown in Table 7, confirm the suggestion of Courtois & Perez (1948) that hard wheats have a higher phytase activity than soft wheats, but the variation in activity between species and between different varieties of one species (i.e. *Triticum vulgare*) is not very great.

Table 7. *Phytase activity of various wheats (1951 harvests)*

(250 mg. samples of wholemeals assayed in buffered substrate, pH 5·15, at 55°.)

Wheat	Phytase activity (μg. P/hr./mg. dry wt.)	Hard or soft
Manitoba grade 1	5·22	Hard
Manitoba grade 2	5·44	Hard
Manitoba grade 3	4·93	Hard
Manitoba grade 4	5·41	Hard
Manitoba grade 5	5·18	Hard
American, Soft White	3·76	Soft
American, No. 2 Red Winter	4·08	Soft
American, Dark Spring	4·88	Hard
English, Pilot	5·22	Hard
English, Holdfast	5·52	Hard
English, Bersee	4·21	Soft
English, Jubiligem	4·76	Soft
Plate	4·54	Soft
Australian	4·06	Soft
Triticum vulgare	4·80*	—
T. polanicus	4·97	—
T. durum	4·28	—
T. spelta	3·82	—
T. turgidum	4·44	—
T. dicoccum	4·47	—

* This figure is the mean of the fourteen figures obtained for the bread wheats.

DISCUSSION

The experiments described in this paper show that the optimum temperature for the hydrolytic activity of phytase is 55°. This figure is comparatively high although, according to Sumner & Somers (1943), it is not unusual for some plant enzymes to have optimum temperatures of activity between 50 and 60°. The experiments on the rate of thermal inactivation of the enzyme show that it is relatively stable and the high temperature for optimum activity is, presumably, partly a reflexion of this thermostability. These temperature relations of wheat phytase have some practical interest since, due to its comparative thermostability, the enzyme will largely retain its activity through the processes of grain drying and of heat treatment where this is practised as a method of improving the milling and baking qualities of wheats. The high optimum

temperature means that, in the making of bread, an appreciable degree of phytase activity occurs during both panary fermentation and the early oven stages. This in turn results in up to 60 % of the phytic acid present in flour being hydrolysed to inositol and free orthophosphate during the baking process (Pringle & Moran, 1942).

Information on the distribution of enzymes between the various anatomical fractions of the wheat grain is of both fundamental and practical interest. Engel (1945, 1947), Engel & Bretschneider (1947) and Engel & Heins (1947), by histological methods using serial sections of the wheat berry, have shown that esterase, proteinase and peptidase activities are found mainly in the embryo, scutellum and aleurone fractions. The data on phytase, recorded in the present paper, appears to be the first to give an approximate quantitative picture of the distributions of an enzyme of the wheat grains. Phytase activity was found to be higher in the scutellum and aleurone fractions and its distribution is thus similar to that of the enzymes studied by Engel. It is also of interest to note that Hinton (1947) and Heathcote, Hinton & Shaw (1952) have shown that these two areas are also the sites of highest concentration of thiamine and nicotinic acid.

With the phytase preparations used in this paper there is, consistently, an initial linear relationship between time and the amount of orthophosphate released. If the course of the hydrolysis of phytate is considered as six consecutive bimolecular reactions with equal velocity constants (k) and if it is assumed that the first step is a 'pseudomonomolecular' reaction, it can be shown that,

$$x = 1 - e^{-kt}\left(1 + \frac{5kt}{6} + \frac{k^2t^2}{3} + \frac{k^3t^3}{12} + \frac{k^4t^4}{72} + \frac{k^5t^5}{720}\right),$$

where x represents the fractions of phytate P released as orthophosphate P in time t. This equation gives a curve which is of the same form as the experimentally observed progress curves. The fit of this theoretical curve is good up to the point of about 65 % phosphate released. Beyond this point the experimental points fall below the theoretical curve. This would be expected if there were inhibition of the enzyme by orthophosphate such as was observed by Luers & Silbereisen (1927) with malt phytase.

SUMMARY

1. Some properties of wheat phytase have been studied using both wholemeal and a partially purified preparation. The optimum pH of the enzyme is 5·15, optimum temperature 55°, Michaelis constant (K_m) $0{\cdot}3 \times 10^{-3}$ M: the enzyme is activated by magnesium ions at an optimum concentration of 0·002 M.

2. A procedure has been worked out for the partial purification (about 20-fold on a dry matter basis) of the enzyme from wholemeal.

3. The distribution of phytase among the anatomical fractions of the wheat grain has been determined.

4. An increase of 6·5-fold in the phytase activity has been observed when a soft wheat (Cappelle Deprez) germinates.

5. The phytase activity of a number of different wheats has been determined, and it has been found that hard wheats have a higher activity than soft wheats.

6. The influence of a number of inorganic and organic salts on phytase action has been determined. Heavy metal salts completely inhibit the enzyme probably by removing the phytate from solution.

7. With the enzyme in solution the rate of thermal inactivation does not follow the unimolecular expression but an approximate value for the heat of inactivation is 41 000 cal.

The author is indebted to Dr J. J. C. Hinton for dissecting certain of the fractions of the wheat grain described in Table 6.

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Serum Esterases

1. TWO TYPES OF ESTERASE (A AND B) HYDROLYSING *p*-NITROPHENYL ACETATE, PROPIONATE AND BUTYRATE, AND A METHOD FOR THEIR DETERMINATION

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Huggins & Lapides (1947) have developed a sensitive method for the determination of esterases using as substrates *p*-nitrophenyl esters of the lower fatty acids. Although these esters are esters of phenols it was assumed that they were hydrolysed by what is usually termed 'ali-esterase'; no evidence was given on this point. It has been shown that other enzymes will hydrolyse *p*-nitrophenyl esters: Zeller, Fleischer, McNaughton & Schweppe (1949) have demonstrated that a purified erythrocyte cholinesterase will hydrolyse *p*-nitrophenyl acetate, and Whittaker (1951) has produced some evidence that both the true and pseudocholinesterases of human blood will hydrolyse a variety of substituted phenyl acetates. It is, therefore, not at all clear what enzymes were being determined in the methods developed by Huggins & Lapides (1947).

During the course of work on the enzyme which will hydrolyse diethyl *p*-nitrophenyl phosphate (E 600) it was found that there are in most sera two quite distinct types of esterase which will hydrolyse *p*-nitrophenyl acetate, propionate and butyrate. One type is inhibited, like cholinesterase, by 10^{-8} M-E 600, while the other is not inhibited at all. This paper describes the work leading to the development of quantitative methods for the estimation of these two types of esterase and which is part of a thesis which has been accepted by the University of London for the degree (external) of Doctor of Philosophy in the Faculty of Science.

METHODS

p-Nitrophenyl acetate, propionate and butyrate were prepared by a procedure essentially the same as described by Huggins & Lapides (1947). *p*-Nitrophenol was esterified by the corresponding acyl chloride in benzene in the presence of magnesium turnings and it was found that far better yields of ester were obtained in benzene saturated with water than in dry benzene. The acetate and propionate were crystallized from methanol. The solubilities of these esters in water at 25° are approximately 10 times less than those given by Huggins & Lapides (1947).

Estimation of esterase activity. Since these esters are unstable, a colorimetric method based on the determination of *p*-nitrophenol is not suitable. A manometric method has been developed, based on the CO_2 liberated from bicarbonate buffer by the 2 moles of acid produced during the hydrolysis. The substrate suspension was prepared by dissolving 100 mg. of the esters in 0·5 ml. of methanol with warming and then blowing the solution rapidly from a Pasteur pipette into 25 ml. of bicarbonate buffer ($NaHCO_3$, 0·031 M; NaCl, 0·162 M; gelatin, 0·1% w/v) and shaking vigorously to obtain a fine suspension of the ester. 1·0 ml. of the enzyme preparation (suitably diluted with the bicarbonate buffer) in the side arm of a Warburg flask was tipped into 3·0 ml. of substrate suspension after the flask had been gassed with 5% (v/v) CO_2 + 95% N_2 and equilibrated. The output of CO_2/min. is calculated from readings taken every 5 min.

from 10 to 40 min. using the method of Aldridge, Berry & Davies (1949). The non-enzymic hydrolysis has been obtained by extrapolating to zero enzyme concentration the straight line obtained when the output of CO_2/min. is plotted against the enzyme concentration.

Throughout this paper the enzyme(s) hydrolysing *p*-nitrophenyl acetate, propionate and butyrate have been termed simply the esterase(s). The cholinesterases have been excluded by their inhibition by eserine.

RESULTS

Enzymic hydrolysis of p*-nitrophenyl esters and its sensitivity to* E 600

It was found that rabbit, rat and horse sera hydrolyse *p*-nitrophenyl acetate, propionate and butyrate actively and under the conditions of determination this activity is such that a 50- to 100-fold dilution of the serum must be used. Rabbit serum hydrolyses the acetate at a higher rate than the butyrate, and for rat serum the reverse is true.

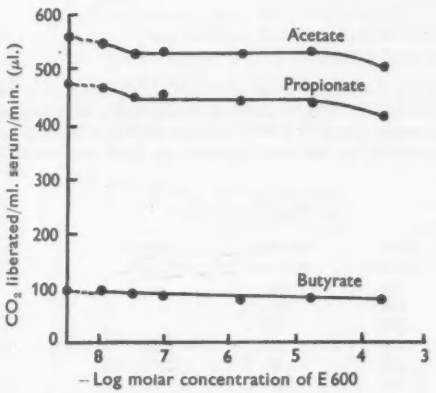

Fig. 1. The hydrolysis of *p*-nitrophenyl acetate, propionate and butyrate by rabbit serum after incubation for 30 min. at 37° with various concentrations of E 600. Original activity determined without inhibitor.

When the sensitivity of these esterases to incubation with E 600 was examined it was found that the hydrolysis of *p*-nitrophenyl acetate by rabbit serum was hardly prevented at all by previous incubation with 10^{-4} M-E 600 while the hydrolysis of *p*-nitrophenyl butyrate by rat serum was nearly stopped by 10^{-8} M-E 600. The hydrolysis of all these *p*-nitrophenyl esters by rabbit, rat and horse sera has therefore been determined after incubation with various concentrations of E 600 at 37° for 30 min. The results are given in Figs. 1–3 which show differences in the sensitivity of the esterase in the rabbit, rat and horse sera towards E 600. In rabbit serum the enzymes are hardly inhibited by concentrations of E 600 as high as 10^{-3} M. In rat serum hydrolysis of all three esters is inhibited by 10^{-7} to 10^{-8} M-E 600. In horse serum hydrolysis of *p*-nitrophenyl propionate and butyrate is strongly inhibited by

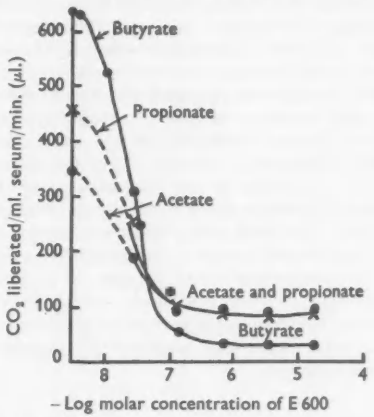

Fig. 2. The hydrolysis of *p*-nitrophenyl acetate, propionate and butyrate by rat serum after incubation for 30 min. at 37° with various concentrations of E 600. Original activity determined without inhibitor.

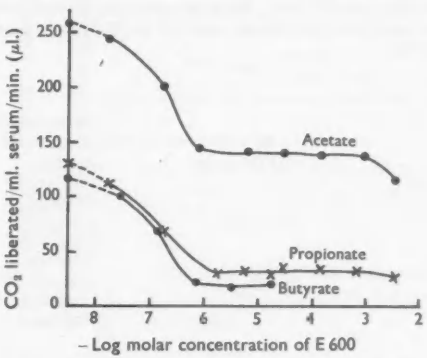

Fig. 3. The hydrolysis of *p*-nitrophenyl acetate, propionate and butyrate by horse serum after incubation for 30 min. at 37° with various concentrations of E 600. Original activity determined without inhibitor.

10^{-6} to 10^{-8} M-E 600, while hydrolysis of *p*-nitrophenyl acetate is only partly inhibited by 10^{-3} M-E 600. These results could be accounted for by the existence of two separate esterases, one inhibited by 10^{-7} M-E 600 and another which is not inhibited by 10^{-3} M-E 600. The data plotted in the graphs (Figs. 1–3) may be used to calculate the rates of hydrolysis of the three substrates for the resistant and the sensitive types of enzyme.

The results of these calculations are given in

Table 1. Inspection of Table 1 and Figs. 1–3 show that in all three sera the resistant type of esterase hydrolyses the acetate more rapidly than the butyrate: in rat serum the sensitive type hydrolyses the butyrate more rapidly than the acetate, while in horse serum the rate of hydrolysis of the three substrates is similar. Individual sera vary to some degree in their content of the two types of esterase.

The predominant esterases in rabbit, rat and horse sera which hydrolyse *p*-nitrophenyl esters may be clearly differentiated by their widely differing sensitivity to E 600. There has been no previous indication in the literature that these esterases may be separated by the effects of selective inhibitors. However, serum lipase and human milk lipase have been shown to be inhibited by tri-*o*-cresyl phosphate and tri-*o*-chlorophenyl phosphate (Bloch, 1943; Hottinger & Bloch, 1943) and Webb (1948) has also demonstrated that liver esterase and human milk lipase are inhibited by di*iso*propyl fluorophosphonate.

Sensitivity of the esterases to other cholinesterase inhibitors

In view of this differing sensitivity of the esterases to E 600, other members of the broad group of organophosphorus inhibitors were tried. Tetraethyl pyrophosphate and di*iso*propyl fluorophosphonate were chosen as representative types of this class of inhibitors. Figs. 4–6 show the inhibition of the same enzyme preparations by tetraethyl pyrophosphate, di*iso*propyl fluorophosphonate and E 600. The enzyme in rat serum was inhibited by low concentrations of each inhibitor. In Table 2 the concentrations of inhibitor giving 50% inhibition (after 30 min. incubation at 37°) are listed together with the values for the inhibition of true and pseudocholinesterase. The values for inhibition of the cholinesterases agree well with those in the literature (Jones, Meyer & Karel, 1948; Mackworth & Webb, 1948; Grob & Harvey, 1949). It is interesting that the sensitivity to inhibition by these inhibitors is of

Table 1. *The presence in rabbit, rat and horse sera of esterases resistant and sensitive to treatment with* E 600

(Enzyme activities are expressed as the output of CO_2/ml. serum/min. calculated from the output of CO_2 obtained on the addition of a suitable dilution of serum to a suspension of the *p*-nitrophenyl ester in the bicarbonate buffer after gassing with 5% (v/v) CO_2 in N_2. Total esterase is the enzyme activity determined without E 600, resistant esterase is the activity after incubation for 30 min. with 10^{-5} M-E 600 and sensitive esterase is the difference between the total and resistant esterase.)

		Enzyme activity (μl. CO_2/ml./min.)		
Material	*p*-Nitrophenyl ester as substrate	Total esterase	Resistant esterase	Sensitive esterase
Rabbit serum	Acetate	565	525	—
	Propionate	475	445	—
	Butyrate	100	80	—
Rat serum	Acetate	340	80	260
	Propionate	460	80	380
	Butyrate	635	30	605
Horse serum	Acetate	255	140	115
	Propionate	130	30	100
	Butyrate	120	20	100

Table 2. *The sensitivity of esterase in rat serum, true cholinesterase in sheep erythrocytes and pseudocholinesterase in horse serum to organophosphorus inhibitors*

(Enzyme preparations were incubated for 30 min. at 37° with the inhibitor prior to the addition of substrate. The concentrations of inhibitor are those during the incubation period. The esterase preparation used was 0·5 ml. rat serum diluted 50-fold; true cholinesterase preparation was 0·5 ml. sheep erythrocytes washed with saline twice and resuspended and diluted to the volume of the original whole blood; pseudocholinesterase preparation was 0·5 ml. horse serum diluted 5-fold. All dilutions were made in the bicarbonate buffer. The results for rat serum are corrected for the small amount of butyrate hydrolysis which is not inhibited by 10^{-5} M inhibitor. True and pseudocholinesterases are completely inhibited by 10^{-5} M inhibitor.)

	Molar concentrations for 50% inhibition		
Inhibitor	Butyrate hydrolysis (rat serum)	True cholinesterase (sheep erythrocytes)	Pseudocholinesterase (horse serum)
Tetraethyl pyrophosphate	$4{\cdot}8 \times 10^{-8}$	$1{\cdot}1 \times 10^{-8}$	—
Di*iso*propyl fluorophosphonate	$4{\cdot}5 \times 10^{-8}$	$4{\cdot}6 \times 10^{-7}$	$2{\cdot}3 \times 10^{-9}$
Diethyl *p*-nitrophenyl phosphate	$1{\cdot}8 \times 10^{-8}$	$2{\cdot}1 \times 10^{-8}$	$5{\cdot}4 \times 10^{-9}$

the same order for cholinesterase and the esterase in rat serum hydrolysing *p*-nitrophenyl butyrate. Fig. 4 shows that with this particular sample of rabbit serum about 15 % inhibition was produced by 10^{-7} M-E 600. This is in agreement with the results obtained with tetraethyl pyrophosphate and di*iso*-

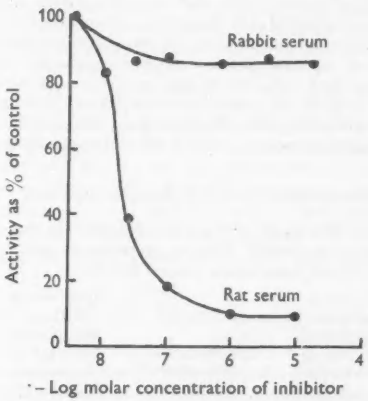

Fig. 4. The hydrolysis of *p*-nitrophenyl acetate by rabbit serum and of *p*-nitrophenyl butyrate by rat serum after incubation for 30 min. at 37° with E 600.

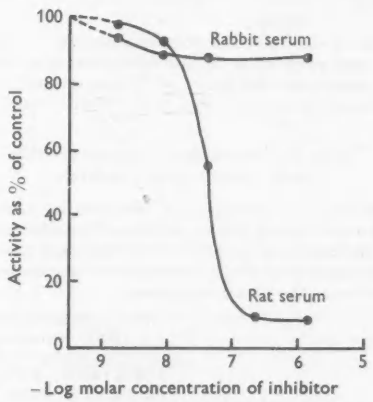

Fig. 5. The hydrolysis of *p*-nitrophenyl acetate by rabbit serum and of *p*-nitrophenyl butyrate by rat serum after incubation for 30 min. at 37° with tetraethyl pyrophosphate.

propyl fluorophosphonate (Figs. 5 and 6) and is due to the presence of a small amount of the sensitive type of the esterase in this sample of serum.

Zeller *et al.* (1949) and Whittaker (1951) have shown that *p*-nitrophenyl acetate is hydrolysed by true and pseudocholinesterase. Eserine is the most satisfactory agent for the differentiation of cholinesterases from the aliphatic esterases (Richter & Croft, 1942; Myers & Mendel, 1949). The results in Table 3 show that neither the rabbit nor rat enzymes are inhibited until a concentration of 10^{-3} M-eserine is reached and then only slightly. With horse serum 14 % inhibition is produced by 10^{-6} M-eserine and this remains constant up to 10^{-4} M. Horse serum contains a high pseudocholinesterase activity (Mendel, Mundel & Rudney, 1943), and Sturge & Whittaker (1950) have shown that a whole range of esters is hydrolysed by a purified cholinesterase from horse serum.

The results in Table 4 are in agreement with the view that the activity against *p*-nitrophenyl esters, which is inhibited by treatment with 10^{-5} M-

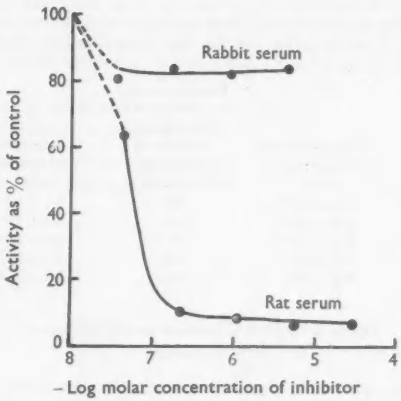

Fig. 6. The hydrolysis of *p*-nitrophenyl acetate by rabbit serum and of *p*-nitrophenyl butyrate by rat serum after incubation for 30 min. at 37° with di*iso*propyl fluorophosphonate.

eserine, is due to the presence of cholinesterases. Any method for the determination of these esterases must take this into account.

Sensitivity of the esterases to other inhibitors

In Table 5 the results are given of an examination of the sensitivity of rabbit and rat serum esterase to copper, nickel and *p*-chloromercuribenzoic acid. The concentrations of rabbit and rat serum used with *p*-nitrophenyl acetate and butyrate as substrates, respectively, are not sufficiently different to cause much error due to non-specific absorption of the inhibitors. The esterase in rabbit serum appears to be more sensitive to metallic inhibitors than the esterase in rat serum. The ratio of the concentration for 50 % inhibition of the rat serum enzyme to that for the rabbit serum enzyme is 10 for copper sulphate and 30 for *p*-chloromercuribenzoic acid.

Inhibition of the rat serum esterase by nickel sulphate could not be determined since a precipitate, presumably of a basic carbonate, was produced when above 4×10^{-4}M-nickel sulphate was used. Utilizing the fact that a preparation of the esterase resistant to E 600 may be made by incubating serum with E 600 (5 μg./ml.) for 30 min. at 37° to destroy all the sensitive enzyme, it has been shown that the hydrolysis of *p*-nitrophenyl acetate by such a preparation from horse serum is also sensitive to *p*-chloromercuribenzoic acid; the concentration for 50 % inhibition is 1×10^{-5}M. The rat serum esterase appears to be slightly more sensitive to sodium fluoride.

of E 600 in absolute ethanol (10 mg./ml.) is diluted to 100 ml. with the bicarbonate buffer. The dilution is prepared freshly every day. (2) Eserine solution, 8×10^{-5}M. This is prepared from a stock solution of eserine sulphate in water (2·6 mg./ml.) by diluting 100 times with the bicarbonate buffer. The dilution is freshly prepared every day.

Procedure. Into two test tubes are placed 5 ml. of a suitable dilution of serum. To one, 5 ml. of the eserine solution are added and to the other, 5 ml. of the E 600 solution. They are incubated at 37° for 30 min. Meanwhile, the buffer and substrates are prepared as previously described and 3 ml. are pipetted into the centre compartment of each Warburg flask. After the 30 min. incubation with eserine or E 600, 1 ml. of the serum solution is pipetted into the side arm of a Warburg flask, after making any necessary dilutions with eserine solution or with buffer in the case of the one

Table 3. *Effect of eserine on the hydrolysis of* p*-nitrophenyl esters by rabbit, rat and horse sera*

(Enzyme preparations were incubated with eserine for 15 min. at 37° prior to the addition of substrate. The concentrations of eserine given are the final concentrations after the addition of substrate. Enzyme preparations were 0·5 ml. rabbit serum diluted 100-fold, 0·5 ml. rat serum diluted 50-fold and 0·5 ml. horse serum diluted 20-fold.)

Concentration of eserine (M)	Rabbit serum with *p*-nitrophenyl acetate as substrate (% activity)	Concentration of eserine (M)	Rat serum with *p*-nitrophenyl butyrate as substrate (% activity)	Concentration of eserine (M)	Horse serum with *p*-nitrophenyl butyrate as substrate (% activity)
$3·9 \times 10^{-3}$	86	$1·1 \times 10^{-3}$	42	$2·6 \times 10^{-3}$	74
$3·9 \times 10^{-4}$	94	$1·1 \times 10^{-4}$	95	$5·2 \times 10^{-4}$	89
$3·9 \times 10^{-5}$	101	$1·1 \times 10^{-5}$	98	$1·04 \times 10^{-4}$	88
$3·9 \times 10^{-6}$	101	$1·1 \times 10^{-6}$	97	$2·1 \times 10^{-5}$	88
$3·9 \times 10^{-7}$	100	$1·1 \times 10^{-7}$	98	$4·2 \times 10^{-6}$	86

Table 4. *Effect of eserine on the esterase in horse serum*

(Enzyme was incubated with eserine for 15 min. at 37° prior to the addition of the substrates. The final concentration of eserine after the addition of substrate was $3·8 \times 10^{-5}$M. Enzyme preparations were 0·5 ml. serum diluted 20-fold with the *p*-nitrophenyl esters as substrates, 0·5 ml. serum diluted 13-fold with the choline esters.)

Substrate	Activity (μl. CO_2/ml./min.) Without eserine	With eserine	Difference
p-Nitrophenyl acetate	229	193	36
p-Nitrophenyl propionate	164	132	32
p-Nitrophenyl butyrate	152	107	45
Acetylcholine	84	Nil	84
Benzoylcholine	29	Nil	29

Method for the determination of the two types of esterase

On the basis of the differing sensitivities of esterase to inhibition by E 600, quantitative methods for the determination of these two distinct types of esterase have been developed.

Reagents. These are identical with those for total esterase estimation given earlier with the addition of: (1) E 600 solution, 10 μg./ml. ($3·6 \times 10^{-5}$M). Stock solution (0·1 ml.) incubated with E 600. The procedure, thereafter, is identical with that given earlier for the determination of esterase. The results are calculated as μl. CO_2/ml. serum/min. The determination using the serum incubated with eserine gives the total esterase (1). The sample incubated with E 600 gives the resistant enzyme (2) (A-esterase). The sensitive esterase (B-esterase) is given by the difference between (1) and (2). (See below for explanation of nomenclature.)

The determination of total esterase must be carried out in

Table 5. *Sensitivity of esterases of rabbit and rat sera to other inhibitors*

(Inhibitor and diluted serum incubated for 15 min. at 37° prior to tipping into the substrate. The substrate contains the same concentration of inhibitor as in the serum during incubation. The concentrations of serum given are those during the 15 min. incubation.)

Inhibitor	Molar concentrations for 50 % inhibition: 1·33 % rabbit serum with *p*-nitrophenyl acetate as substrate	2·0 % rat serum with *p*-nitrophenyl butyrate as substrate
Nickel sulphate	$1·3 \times 10^{-5}$	—
Copper sulphate	$1·4 \times 10^{-5}$	$1·6 \times 10^{-4}$
p-Chloromercuribenzoic acid	$1·4 \times 10^{-5}$	4×10^{-4}
Sodium fluoride	8×10^{-3}	$2·3 \times 10^{-3}$

the presence of eserine in order to prevent the hydrolysis of *p*-nitrophenyl esters by cholinesterase. Since eserine is a reversible inhibitor any dilutions of the original incubation mixture must be made with solutions containing eserine and the final concentration in the flask should be approximately 10^{-5} M. After addition of E600, which produces an irreversible inhibition *in vitro*, the solutions may be diluted with buffer. The sensitive esterase is almost completely inhibited by 10^{-7} M solutions of E600. It is necessary, therefore, to be very careful to avoid the accidental contamination of solutions of the enzymes by the use of pipettes that have been used for dilutions of E600.

For convenience and clarity we have used a simple method of nomenclature for these enzymes. The substances which both of these enzymes are hydrolysing are esters and both are, therefore, called esterases. The observation that the enzyme not inhibited by E600 hydrolysed acetate at a higher rate than butyrate suggested that this enzyme be called A-esterase and the sensitive enzyme, therefore, B-esterase. This avoids placing any emphasis on their specificity to a particular substrate.

A-esterase is, therefore, the esterase which is not inhibited by E600, and B-esterase is the esterase which is inhibited by E600.

A- and B-esterases in various sera

Sera from various species have been examined using the methods for the determination of A- and B-esterase. The list is rather an arbitrary collection and in many cases only one sample of serum has been examined (Fig. 7). However, it is shown that the methods have a general application, but the finer differentiation of substrate-specificity patterns is not possible with such a small number of samples from each species. With one exception A-esterase is present in the sera from thirteen species. Duck serum has no detectable A-esterase activity and fowl serum has very little. In some sera, e.g. of the sheep, cow and goat, only A-esterase appears to be present. Sera from the rabbit sometimes contain only A-esterase but usually there is some B-esterase present. B-esterase is present in the sera of the cat, guinea pig, rat, horse and rabbit.

All the A-esterases demonstrated in the sera examined hydrolyse *p*-nitrophenyl acetate at a higher rate than the butyrate under the conditions of determination. The substrate patterns of the A-esterase from different species vary considerably, for instance A-esterase from rabbit serum hydrolyses the propionate at roughly 75 % of the rate of the acetate, while the A-esterase of the horse, sheep and cow hydrolyses the propionate at only 30 % of the rate of the acetate. In two species, the ferret and the cat, the propionate is hydrolysed at a higher rate than the acetate. Even with these differing

Table 6. *Summation experiments on A-esterase of rabbit serum using* p*-nitrophenyl butyrate, and ethyl acetate and butyrate as substrates*

(A-esterase prepared by incubation of a suitable dilution of rabbit serum with 5 μg. E600/ml. for 30 min. at 37° prior to summation experiments. Substrate concentrations for ethyl acetate and butyrate were 0·4 ml./25 ml. bicarbonate buffer and for *p*-nitrophenyl butyrate suspension, 4 mg./ml.)

Enzyme activities (μl. CO_2/ml./min.)				
p-Nitrophenyl butyrate (*a*)	Ethyl acetate (*b*)	*p*-Nitrophenyl butyrate + ethyl acetate (*c*)	(*a*) + (*b*)	(*c*) − (*a*)
65·6	8·4	51·2	74·0	−14·4
64·1	7·6	49·9	71·7	−14·2
p-Nitrophenyl butyrate (*a*)	Ethyl butyrate (*b*)	*p*-Nitrophenyl butyrate + ethyl butyrate (*c*)	(*a*) + (*b*)	(*c*) − (*a*)
58·4	0	47·5	58·4	−10·9
55·4	0	37·7	55·4	−17·7

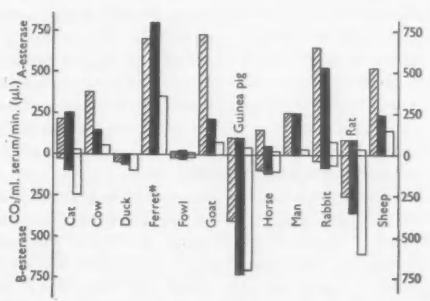

Fig. 7. Histogram of the rates of hydrolysis of *p*-nitrophenyl esters by the sera of various species. Substrates: ▨, *p*-nitrophenyl acetate; ■, propionate; □, butyrate. * For convenience the rates of hydrolysis plotted for ferret serum are one-half the rates actually determined.

specificity patterns no exceptions have so far been found to the general rule that A-esterase hydrolyses the acetate at a higher rate than the butyrate.

Hydrolysis of aliphatic esters by A-esterase

Huggins & Lapides (1947) assumed that the enzyme which hydrolyses *p*-nitrophenyl acetate, propionate and butyrate, is the same as that which hydrolyses aliphatic esters such as ethyl acetate. No evidence is given in their paper for this point of view. Summation experiments have been carried out on the A-esterase of the rabbit using *p*-nitrophenyl butyrate, and ethyl acetate and butyrate as substrates. The results are given in Table 6 and indicate that there is competition between *p*-nitrophenyl butyrate and ethyl acetate for A-esterase. If ethyl butyrate was hydrolysed the amount was too small to measure, but some lowering of the rate of hydrolysis of *p*-nitrophenyl butyrate was caused by the presence of this compound. This probably indicates competition for the same active centre by both esters.

DISCUSSION

With the aid of E 600 it has been demonstrated that the esterases present in many sera may be divided into two distinct types. One type (A-esterase) is not inhibited by E 600 up to concentrations of 10^{-3} M, whereas the other type (B-esterase) is extremely sensitive to E 600 and is inhibited by concentrations as low as 10^{-8} M. E 600 is an excellent inhibitor for this purpose. It is easy to prepare pure, is relatively stable to hydrolysis and is extremely active. It is clear that the organophosphorus inhibitors are by no means specific inhibitors for cholinesterase even though this action may govern their toxicity. Chymotrypsin is inhibited by di*iso*propyl fluorophosphonate (Jansen, Nutting, Jang & Balls, 1949) and by E 600 (Hartley & Kilby, 1952). Acetylesterase from oranges is inhibited by tetraethyl pyrophosphate and di*iso*propyl fluorophosphonate (Jansen, Nutting & Balls, 1948). In this paper it has been shown that B-esterase from rat serum is inhibited by E 600, tetraethyl pyrophosphate and di-*iso*propyl fluorophosphonate.

The A-esterase in the sera examined hydrolyses *p*-nitrophenyl acetate at a higher rate than the butyrate. B-esterases appear to hydrolyse the butyrate at the same or a higher rate than the acetate, though caution must be used since we have as yet no evidence whether the B-type of esterase includes other esterases known as lipase. It should be noted that the relative rates of hydrolysis of substrates refer only to the conditions of determination used in this paper. The relative amounts of A- and B-esterase vary from one animal to another. For example, the A-esterase activity against *p*-nitrophenyl acetate in sera from four rabbits varied from 660 to 521 μl. CO_2/ml./min., while the B-esterase activity varied from 0 to 300 μl. CO_2/ml./min. With such variation in the amounts of two esterases in each serum, a study of their total activity in serum with substrates alone must, therefore, give misleading results. The apparent specificity pattern will vary from sample to sample. A combination of the use of inhibitors of the E 600 type and substrates will probably help to solve the problem of the specificity of mixtures of esterases.

If the activity of A-esterase of rabbit serum acting on *p*-nitrophenyl acetate is taken as 100 then its activity against the butyrate is 16. Since, in Table 6, this A-esterase has been shown to hydrolyse *p*-nitrophenyl butyrate approximately 8 times faster than ethyl acetate, *p*-nitrophenyl acetate will therefore be hydrolysed by this enzyme approximately 40–50 times faster than ethyl acetate. Before it can be decided whether the activity called ali-esterase is identical with that hydrolysing *p*-nitrophenyl esters, much more work requires to be carried out on purified preparations of A- and B-esterases from different species. It can be said, however, that other enzymes hydrolyse aromatic esters at a higher rate than aliphatic esters, e.g. the phosphatases (King & Delory, 1939).

It is very difficult to decide the function of an enzyme and especially of those with a low specificity. Among the sera we have examined those from the ruminants (cow, sheep and goat) all hydrolyse the acetate at a much higher rate than the propionate or the butyrate. It is known that large amounts of acetic acid are produced in the rumen by the digestion of cellulose (Stephenson, 1949; Gray, Pilgrim, Rodda & Weller, 1951). It is tempting to speculate that there is some connexion between these two facts.

SUMMARY

1. Serum esterases of many species may be separated into two distinct types, A- and B-esterase.

2. The A-type is not inhibited by E 600 and hydrolyses *p*-nitrophenyl acetate at a higher rate than *p*-nitrophenyl butyrate.

3. The B-type is inhibited by 10^{-7} to 10^{-8} M-E 600 and hydrolyses *p*-nitrophenyl butyrate at the same or a higher rate than *p*-nitrophenyl acetate.

4. It is possible that both the A-type and B-type esterases contribute to the ali-esterase activity of serum.

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Serum Esterases

2. AN ENZYME HYDROLYSING DIETHYL *p*-NITROPHENYL PHOSPHATE (E 600) AND ITS IDENTITY WITH THE A-ESTERASE OF MAMMALIAN SERA

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It has been stated (Roche, 1950): 'The orthophosphoric tri-esters which are easily hydrolysed by acids, bases or even boiling water, are not hydrolysed enzymatically.' If a solution of diethyl *p*-nitrophenyl phosphate (E 600) is added to rabbit serum the serum rapidly turns yellow. This is due to the liberation of *p*-nitrophenol by the hydrolysis of E 600.

E 600 is an orthophosphoric tri-ester and has been studied primarily for its anticholinesterase properties. It is a very active inhibitor (Aldridge, 1950; Aldridge & Davison, 1952) and will inhibit the cholinesterase activity of sheep erythrocytes by 50% after incubation at 37° for 30 min. at a concentration of 2×10^{-8} M. It is also very toxic, the LD_{50} for rats being about 0·5 mg./kg. It is therefore of interest that this substance can be hydrolysed in mammals. In this paper, methods for the determination of this enzyme (E 600-esterase) are given followed by a study of its properties and distribution in some species.

It has been shown (Aldridge, 1953) that there are two distinct types of esterase which will hydrolyse *p*-nitrophenyl acetate, propionate and butyrate. One esterase (A-esterase) is not inhibited by E 600 while the other (B-esterase) is inhibited by concentrations as low as 10^{-8} M. In this paper evidence will be presented which shows that the enzyme hydrolysing E 600 and A-esterase are identical enzymes. A preliminary report of these observations has been published (Aldridge, 1951).

The work described in this paper is part of a thesis which has been accepted by the University of London for the degree (external) of Doctor of Philosophy in the Faculty of Science.

EXPERIMENTAL

Materials

Diethyl *p*-nitrophenyl phosphate was first prepared by Schrader (1947) who called it E 600. It is a pale yellow liquid, sp.gr. 1·27, practically non-volatile at room temperature and atmospheric pressure, b.p. 160° at 0·05 mm. Hg pressure. It is soluble in water at 25–37° to 2·37 mg./ml. and is slowly hydrolysed in buffers at pH 7·8 to give diethyl phosphoric acid and *p*-nitrophenol, no orthophosphate being produced. The first-order constant for its hydrolysis at 37° in Sørensen's M/15 phosphate buffer, pH 7·6, is $3·9 \times 10^{-5}$ (min.$^{-1}$), the half life under these conditions being 11·2 days. I am grateful to Mr B. Topley (Albright and Wilson Ltd.) for a generous supply of E 600.

Manometric method of esterase estimation

Since two molecules of acid are liberated for every molecule of E 600 hydrolysed it is possible to use a method similar to that developed by Ammon (1933) for the determination of cholinesterase.

Reagents. (*a*) Buffer. $NaHCO_3$, 0·031 M; NaCl, 0·162 M; gelatin, 0·1% (w/v). When gassed with 5% (v/v) CO_2 in 95% N_2 a solution of pH 7·73 at 37° is obtained. (*b*) Buffer-substrate. 4 drops E 600 (approx. 80 mg.) are dissolved in 0·5 ml. methanol and then blown rapidly from a Pasteur pipette into 25 ml. buffer.

Procedure. The output of CO_2 is determined after tipping 0·5 ml. of a suitable dilution of enzyme into 3·5 ml. buffer-substrate. The results are calculated using the simplified linear regression method of Aldridge, Berry & Davies (1949). A linear output of CO_2 with respect to time and the output of CO_2/min. is a linear function of the enzyme concentration. Without the addition of gelatin there is a slight loss of activity during the determination. The determination of A-esterase is similar to the above and has been described in detail (Aldridge, 1953).

Colorimetric method

p-Nitrophenol liberated during the hydrolysis of E 600 has an intense yellow colour at pH 7 or above. A colorimetric method has been developed for use in the routine determination of the enzyme distribution in tissues. *p*-Nitrophenol has been extracted from the highly coloured and turbid suspensions with a 1:1 (v/v) mixture of *n*-butanol and toluene.

Reagents. (*a*) Sørensen's M/15 phosphate buffer, pH 7·6. (*b*) E 600, which should be free from uncombined *p*-nitrophenol. An approximately 10% (v/v) solution of E 600 in $CHCl_3$ is washed with equal volumes of 2% (w/v) $NaHCO_3$ until only a very pale yellow aqueous layer is obtained. All the bicarbonate washings are united and washed with 15 ml. $CHCl_3$. All the $CHCl_3$ washings are united and washed once with water and once with 1% (w/v) citric acid solution. The $CHCl_3$ is run through a filter paper, dried with anhydrous $CaSO_4$ and the $CHCl_3$ evaporated in a stream of dry air. (*c*) Buffer-substrate. One drop (about 20 mg.) of purified E 600 is added to 10 ml. buffer. It is shaken vigorously. (*d*) Butanol/toluene mixture. Equal volumes are mixed. (*e*) Ammonia (sp.gr. 0·880) and absolute ethanol (1:4, v/v). (*f*) Hydrochloric acid, approx. 5 N. (*g*) Gelatin 2% (w/v).

Procedure. Into two tubes *A* and *B* 9·5 ml. of buffer-substrate are pipetted and warmed to 37°. In a separate tube, serum or tissue homogenate suitably diluted with 2% (w/v) gelatin are also warmed. To tube *A* 0·5 ml. of enzyme preparation is added, mixed and left at 37°. After 30 min. 0·5 ml. of 5 N-HCl is added to both tubes followed by 0·5 ml. of enzyme preparation to tube *B*. To each, 10 ml. of butanol/toluene mixture are added and shaken vigorously. After separation has taken place not more than 5 ml. of the solvent layer are pipetted off. Just before reading at 420 mμ. 3 ml. of 20% (v/v) ammonia in ethanol are added and the volume adjusted to 10 ml. with butanol/toluene mixture. The *p*-nitrophenol present is read off from a calibration curve prepared in a similar way. $A - B = \mu g.$ *p*-nitrophenol released by 0·5 ml. of enzyme preparation in 30 min. The activity of the preparation is expressed as mg. *p*-nitrophenol liberated in 30 min. at 37° by 1 g. or 1 ml. of tissue. Under these conditions the amount of *p*-nitrophenol liberated is a linear function of enzyme concentration.

Correlation between manometric and colorimetric methods. During the hydrolysis of E 600, 322 μl. CO_2 liberated in the manometric method are equivalent to 1 mg. *p*-nitrophenol. Therefore mg. *p*-nitrophenol/g./30 min. $\times 10{\cdot}7 \equiv \mu l.$ CO_2/ml./min.

A-esterase free from B-esterase and cholinesterase activities may be prepared by incubation with 5 μg./ml. E 600 for 30 min. at 37°. Under these conditions both B-esterase and the cholinesterases are completely inhibited.

RESULTS

E 600-*esterase*

Enzymic nature of hydrolysis of E 600. The hydrolysis of E 600 by rabbit serum is continuous. The rate of this hydrolysis is much higher than the aqueous hydrolysis of E 600 and is proportional to the amount of serum taken. Heating for 5 min. at 68° completely prevents the catalysis of the hydrolysis of E 600 while after 5 min. at 50° it is slightly affected. After dialysis for 2 days at 40° against phosphate buffer, pH 7·6, the solution, although considerably diluted, showed the same activity/mg. of protein as the original preparation. It was concluded that this hydrolysis was an enzymic process. Throughout this paper the enzyme will be called E 600-esterase for, as will be shown later, the enzyme, although it hydrolyses a tri-ester of phosphoric acid, is not related to the phosphatases and has the properties of an esterase.

Table 1. pH *optima of E* 600-*esterase in phosphate buffer*

(Enzyme preparation; 0·5 ml. of 100-fold diluted rabbit serum, M/15 Sørensen phosphate buffers of various pH values. Activity determined by the colorimetric method.)

pH	*p*-Nitrophenol liberated in 30 min. (μg.)
8·2	56
8·0	72
7·8	82
7·6	85
7·4	87
7·2	73
7·0	61
6·8	33
6·6	24

Since rabbit serum is a particularly potent and convenient source of the enzyme most of the work to follow has been carried out using this preparation. In Table 1 is shown the effect of varying the pH on the activity of the enzyme. The pH optimum is between 7·4 and 7·6 and appears to be the same for phosphate, borax-boric acid, veronal-acetate and bicarbonate-carbon dioxide buffering systems.

However, veronal-acetate appears to lower the activity roughly 35 %. The variation of the rate of hydrolysis with different concentrations of substrate is given in Fig. 1. The results have been plotted according to the procedure of Lineweaver & Burk (1934) and thus evaluated K_m is $4{\cdot}5 \times 10^{-4}$ M. This value indicates a fairly high affinity of E 600-esterase for its substrate. The sensitivity of E 600-esterase to the inhibitors nickel sulphate, copper sulphate and *p*-chloromercuribenzoic acid has been determined and the results are shown in Table 2. It is clear that this enzyme is extremely sensitive to these inhibitors. This inhibition by nickel and *p*-chloromercuribenzoic acid is reversed by glutathione and cysteine. E 600-esterase is only inhibited 27 % after incubation with 9×10^{-3} M-sodium iodoacetate

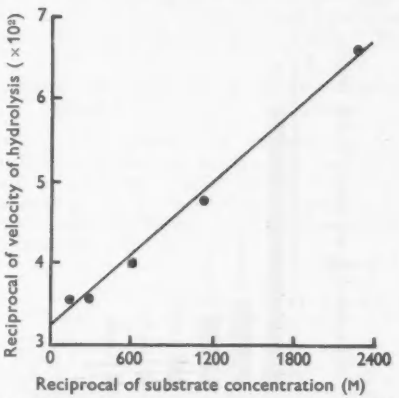

Fig. 1. The relation between the concentration of E 600 and its hydrolysis by rabbit serum. The 'initial velocity' of hydrolysis is given as μl. CO_2 liberated during the first 10 min. The value calculated for K_m from these results is $4{\cdot}5 \times 10^{-4}$ M.

for 30 min. at 37°. Iodoacetate is known to vary in its action on —SH groups (Dixon, 1948). The evidence is therefore suggestive, but by no means conclusive, that E 600-esterase requires —SH groups for its activity. Eserine, which is the best available inhibitor to differentiate ali-esterase from the cholinesterases (Richter & Croft, 1942; Myers & Mendel, 1949), has practically no effect on E 600-esterase, causing only 8 % inhibition at 8×10^{-4} M.

Table 3 shows the results of a crude fractionation of rabbit serum by the procedure of Cohn *et al.* (1950) at $-5°$ and by ammonium sulphate at $+5°$. These two sets of results are in striking contrast for, whereas using Cohn's method the enzyme proteins came down in the globulin fraction, using ammonium sulphate E 600-esterase is still mostly in solution even in 80 % saturated solutions, i.e. the albumin fraction. The possibility must be considered, and particularly with Cohn's method, that protein interactions between various components of the mixture have caused precipitation at unexpected stages of the separation.

Distribution of E 600-esterase in tissues. Aromatic nitro compounds are reduced by enzyme systems to amino compounds (Williams, 1947; Bueding & Joliffe, 1946; Westfall, 1943; Smith & Worrell, 1950; Egami, Ebata & Sato, 1951; Parker, 1952). Such reductions are prevented by the addition of cyanide (Egami *et al.* 1951; Parker, 1952). There are, therefore, two points to consider in the determination of E 600-esterase in tissue homogenates. First, *p*-nitrophenol produced by the hydrolysis of E 600 might be partially reduced, and secondly, the substrate, E 600, might be depleted by its reduction. These possibilities have been examined and it has been shown that there is no significant loss of *p*-nitrophenol from rat-liver homogenate after incubation for 30 min. and that the same E 600-esterase activity is found whether the determination is carried out with or without 10^{-2} M-KCN. It has been concluded that the method as described is suitable for the determination of E 600-esterase in tissue homogenates.

Table 2. *Inhibition of E 600-esterase by metal inhibitors*

(For both methods of determination of activity, the enzyme was incubated with the inhibitor for 15 min. at 37° before adding the substrate.)

Inhibitor	Molar concentration of inhibitor to produce 50 % inhibition	
	Colorimetric method	Manometric method
Nickel sulphate	$7{\cdot}6 \times 10^{-6}$	$1{\cdot}4 \times 10^{-5}$
Copper sulphate	$8{\cdot}9 \times 10^{-6}$	4×10^{-5}
p-Chloromercuribenzoic acid	$4{\cdot}0 \times 10^{-6}$	$1{\cdot}8 \times 10^{-5}$

The results of an examination of the distribution of E 600-esterase in the rabbit, rat and in various sera are given in Figs. 2–4 respectively. The outstanding feature in the rabbit (Fig. 2) is the high activity of the serum. Such a high activity makes the assessment of tissue activity difficult. For example, unperfused liver hydrolysed 9 mg. *p*-nitrophenol g./30 min. while perfused liver hydrolysed 3 mg. In the rat (Fig. 3) the serum activity is low and the tissue with the highest activity is the liver. Pancreas, brain and submaxillary gland which contain high concentrations of hydrolytic enzymes have no appreciable E 600-esterase activity. Amongst the sera examined (Fig. 4) rabbit serum is the most active, followed by that of ferret and sheep. Every serum tested had a measurable enzyme activity even though it was low in many cases.

Table 3. *Fractionation of rabbit serum by the method of Cohn* et al. (1950) *and by ammonium sulphate*

(For Cohn's method the precipitations were carried out at $-5°$ and centrifuged at $+5°$. Using ammonium sulphate both precipitation and centrifuging were done at $+5°$. The precipitates were dissolved in buffer containing 4×10^{-3}M-cysteine. E600-esterase was determined colorimetrically, and protein by the colorimetric biuret method of Robinson & Hogden (1940).)

Fraction	Enzyme activity (mg. *p*-nitrophenol/ 100 mg. protein/30 min.)	Total enzyme activity recovered (mg. *p*-nitrophenol/30 min.)	
Method of Cohn *et al.* (1950)			
I+II+III	59	1150	
IV+V	4·9	204	1354
VI	Nil	Nil	
Original serum	20·7	1332	
Ammonium sulphate fractionation			
40% saturation (ppt.)	6·9	35	
80% saturation (ppt.)	27·2	147	1003
80% saturation (supernatant)	26·8	821	
Original serum	20·2	1110	

E600-esterase and other enzymes in rabbit serum. E600-esterase may be clearly differentiated from the cholinesterases by their differing sensitivities to eserine and to organophosphorus inhibitors. Rabbit serum, which contains the highest E600-esterase activity of any tissue, also contains a number of

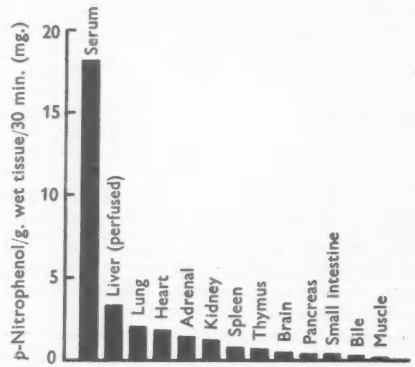

Fig. 2. E600-esterase activity of tissues of the rabbit. With the exception of liver, which was perfused, tissues were rinsed in saline prior to homogenization with a Folley-type homogenizer in M/15 phosphate buffer, pH 7·6, containing 2% (w/v) gelatin. After suitable dilution with buffer-gelatin solution 0·5 ml. was taken for the determination of activity by the colorimetric method.

interesting enzymes which will hydrolyse atropine, monoacetylmorphine, diacetylmorphine (heroin) and an enzyme distinct from pseudocholinesterase which will hydrolyse benzoylcholine but not acetylcholine. It has been shown that the activity against atropine (Bernheim & Bernheim, 1938; Glick & Glaubach, 1941), monoacetylmorphine (Wright, 1942) and benzoylcholine (Ellis, 1947) is only

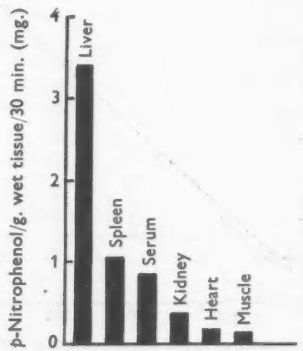

Fig. 3. E600-esterase activity of rat tissues. Method of determination as given in Fig. 2. The activity of pancreas, brain and submaxillary gland was undetectable.

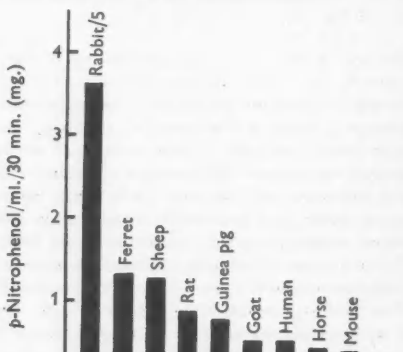

Fig. 4. E600-esterase activity of sera of various species. Method of determination as given in Fig. 2. The activity of rabbit serum is five times the value plotted.

present in the sera of some but not all rabbits. E 600-esterase has never been absent from the sera of over one hundred rabbits. E 600-esterase may be further differentiated from these enzymes and from procaine esterase (Kisch, Koster & Strauss, 1943) by the fact that they are all inhibited by 10^{-5}M-eserine, procaine esterase (Legge & Durie, 1942; Kisch, 1943), monoacetylmorphine and atropine esterase (Wright, 1941; Glick, 1942) and benzoylcholinesterase (Ellis, 1948). The three latter enzymes are inhibited by tetraethyl pyrophosphate (Ellis, 1948).

E 600-esterase and phosphatase activity. Since E 600 is an ester of phosphoric acid the possibility has been examined that E 600-esterase is identical with acid or alkaline phosphatases. It has been shown (Fig. 2) that rabbit kidney, small intestine and bile which have a high alkaline phosphatase activity are low in E 600-esterase activity. Rabbit serum which has a high E 600-esterase activity has a low alkaline phosphatase activity. Spleen, which contains a high acid phosphatase activity, is very low in E 600-esterase. A comparison has been made between the hydrolysis of E 600 and *p*-nitrophenyl phosphate by three tissues of the rabbit. The results in Table 4 show that E 600-esterase cannot be identical with either of the phosphatases.

Table 4. *Hydrolysis of E 600 and* p-*nitrophenyl phosphate by the tissues of the rabbit*

(*p*-Nitrophenol liberated was determined colorimetrically. Sørensen's phosphate buffer, pH 7·6, used for E 600-esterase and a barbitone buffer, pH 7·6, for *p*-nitrophenyl phosphate hydrolysis. 1 unit of enzyme is that amount of enzyme which will liberate 1 mg. of *p*-nitrophenol in 30 min. at 37°.)

Tissue	E 600-esterase (units/ml. or g.)	*p*-Nitrophenyl phosphate hydrolysis (units/ml. or g.)
Serum	24·0	0·02
Bile	Nil	0·04
Kidney* (1)	0·64	3·4
Kidney* (2)	0·13	9·03

* Two different animals.

E 600-esterase and A-esterase

In a previous paper (Aldridge, 1953) it has been demonstrated that there is present in the sera of the rabbit, rat and horse an enzyme (A-esterase) which hydrolyses *p*-nitrophenyl acetate, propionate and butyrate but which is not inhibited by E 600 or similar inhibitors. Since E 600 is a tri-ester of phosphoric acid it is virtually an uncharged molecule and is similar in this respect to *p*-nitrophenyl acetate, propionate and butyrate. Both E 600-esterase and A-esterase are sensitive to nickel, copper and *p*-chloromercuribenzoic acid and neither is inhibited by eserine. The possibility that E 600-esterase and A-esterase are identical has been examined using as enzyme preparations, rabbit, rat and horse sera.

Summation experiments. The substrates *p*-nitrophenyl acetate and E 600 and also *p*-nitrophenyl butyrate and E 600 have been used for these experiments. When the acetate was used, the experiments were carried out using saturated solutions with no substrate in suspension. This procedure was necessary since when a suspension of E 600 was added to a suspension of *p*-nitrophenyl acetate an immediate coagulation of the acetate occurred. It was therefore probable that replacement of the hydrolysed acetate by solution from this coagulum was slow. The results of these experiments are given in Figs. 5–7, and those using *p*-nitrophenyl butyrate and E 600 are given in Table 5. Both series of experiments are consistent with a competition of the enzyme active centre for both substrates.

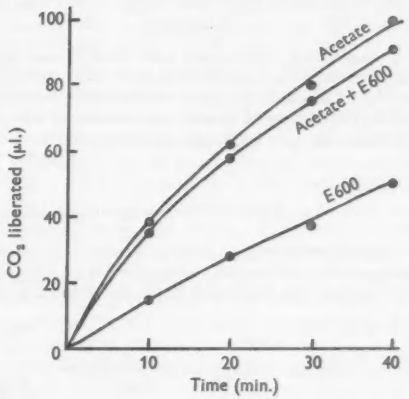

Fig. 5. Summation experiments with rabbit serum using *p*-nitrophenyl acetate and E 600 as substrates. Saturated solutions of substrates in the bicarbonate buffer were used and 0·5 ml. of 100-fold diluted rabbit serum as enzyme. Substrate used shown against each curve.

Ratio of A-esterase to E 600-esterase activities for different preparations. This ratio has been determined for samples of sera from different animals. The sera of the three species, rabbit, rat and horse have been examined. The results (Table 6) show that this ratio is constant within a species and support the idea that A-esterase is the enzyme which hydrolyses E 600. It is interesting that there is a marked difference between the ratio from one species to another. An enzyme preparation purified by Cohn's method (cf. Table 3, fraction I + II + III) gave the same ratio.

Inhibition of E 600-esterase and A-esterase by diethyl phenyl phosphate. Diethyl phenyl phosphate

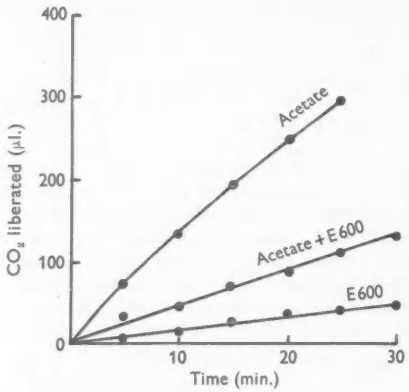

Fig. 6. Summation experiments with horse serum using *p*-nitrophenyl acetate and E600 as substrates. Saturated solutions of substrates in the bicarbonate buffer were used and 0·5 ml. of fivefold diluted horse serum as enzyme. The substrates used are shown against each curve.

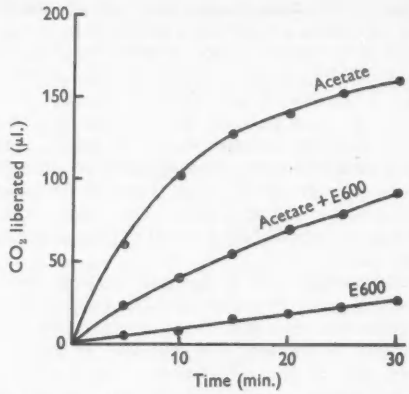

Fig. 7. Summation experiment with rat serum using *p*-nitrophenyl acetate and E600 as substrate. Saturated solutions of substrates in the bicarbonate buffer were used and 1·0 ml. of fivefold diluted rat serum as enzyme. Substrate used shown against each curve.

Table 5. *Summation experiments using* p*-nitrophenyl butyrate and E*600

(A-esterase preparation. A suitable dilution of the serum incubated with 5 µg./ml. of E600 for 30 min. at 37° to remove B-esterase and cholinesterase. A negative sign for (*c*) − (*b*) or (*c*) − (*a*) indicates that with both substrates present the output of CO_2 was less than the greater of the two substrates separately.)

	Enzyme activity (µl. CO_2/ml./min.)				
Serum	*p*-Nitrophenyl butyrate (*a*)	E600 (*b*)	E600 + butyrate (*c*)	(*a*) + (*b*)	(*c*) − (*b*) or (*c*) − (*a*)
Rabbit	66	94	72	160	−22
	60	90	67	150	−23
	58	89	65	147	−24
Rat	30	11	15	41	−15
	28	10	15	38	−13
	29	13	18	42	−11
Horse	18	8	16	26	− 2
	20	8	16	28	− 4

is very stable to hydrolysis. Its half life at pH 7·6 at 37° in M/15 phosphate buffer is 8·2 years (Aldridge & Davison, 1952) and it is approximately 250 times more stable than E600. Since it has a chemical structure very similar to E600 it is reasonable to assume that it will 'fit' the active centre of E600-esterase. If E600-esterase is identical with A-esterase then this compound should also inhibit the hydrolysis of *p*-nitrophenyl acetate. The results in Fig. 8 are consistent with this view. Experiments with A-esterase from rat and horse sera show that in the presence of $2{\cdot}8 \times 10^{-2}$ M-diethyl phenyl phosphate 65 and 63% inhibition of activity, respectively, was obtained.

Table 6. *Ratio of A-esterase and E*600*-esterase activities for the sera of the rabbit, rat and horse*

(A-esterase preparation. B-esterase and cholinesterase activity was prevented by incubation of a suitable dilution of serum with 5 µg./ml. E600 for 30 min. at 37°. The enzyme activities were determined by the manometric methods. Substrate suspensions were for E600, 3 mg./ml., and for *p*-nitrophenyl acetate, 4 mg./ml. Enzyme activities are expressed as µl. CO_2/ml./min.)

Serum	No. of samples	Ratio: $\frac{\text{A-esterase}}{\text{E600-esterase}}$ ± S.D.
Rabbit	10	4·03 ± 0·14
Rat	6	12·2 ± 0·42
Horse	6	18·8 ± 0·81

DISCUSSION

An enzyme has been found which will hydrolyse the tri-ester of phosphoric acid, E 600. This work disproves the statement of Roche (1950) that such esters are not hydrolysed enzymically. It has been demonstrated that by three tests E 600-esterase is identical with an enzyme hydrolysing *p*-nitrophenyl acetate which is not inhibited by E 600 (A-esterase). The sera of the rabbit, rat and horse have been examined and, although E 600-esterase is the same as A-esterase in each species, the enzymes vary from species to species (Table 6). It is becoming clear that enzymes of the same type from different species may have a different spectrum of activities against a number of substrates. This is clear from recent work on cholinesterases from human and horse sera (Mounter & Whittaker, 1950) and from rat heart (Ord & Thompson, 1951). Such differences in proteins from different species are to be expected. Species differences in proteins have been long studied immunologically, and it is well known that not only do the haemoglobins from different species each have their own crystalline form (Reichert & Brown, 1909), but also show functional differences in their affinities for oxygen and carbon monoxide (Anson, Barcroft, Mirsky & Oinuma, 1924). Once species specificity of enzymes is accepted the commonly used method of testing the identity of two activities by comparing the order of the rates of hydrolysis of two substrates by several enzyme preparations from different species, is no longer a valid procedure. On such a procedure Kisch *et al.* (1943) deduced that procaine esterase was not identical with pseudocholinesterase. However, the careful work of Kalow (1952) has shown that these two activities are mediated by the same enzyme. These two conflicting conclusions can be explained by assuming that the ratio of the rates of hydrolysis of procaine and acetylcholine will vary from species to species with the order of activities also varying.

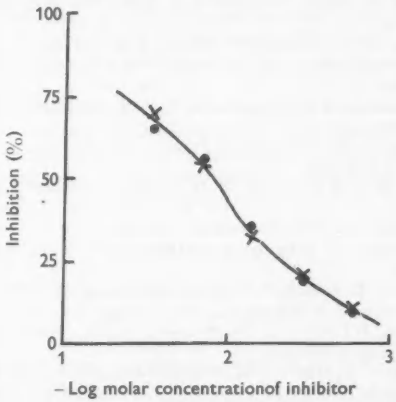

Fig. 8. Inhibition of A-esterase and E 600-esterase of rabbit serum by diethyl phenyl phosphate. A-esterase preparation was a suitable dilution of serum incubated with 5 μg./ml. E 600 for 30 min. at 37°. Saturated solutions of *p*-nitrophenyl acetate and E 600 were used as substrates for A-esterase and E 600-esterase respectively. To these were added varying amounts of a solution saturated with the substrate and diethyl phenyl phosphate. ×, A-esterase; ●, E 600-esterase.

Several enzymes are known to hydrolyse nitrophenyl esters, erythrocyte cholinesterase (Zeller, Fleischer, McNaughton & Schweppe, 1949), human serum cholinesterase (Whittaker, 1951), acetyl esterase from oranges (Jansen, Nutting & Balls, 1948), chymotrypsin (Hartley & Kilby, 1952) and A- and B-esterase of many sera (Aldridge, 1953). Five of the above six enzymes are inhibited by organophosphorus compounds, chymotrypsin (Jansen, Nutting, Jang & Balls, 1949*a*; Jansen, Curl & Balls, 1951; Hartley & Kilby, 1950), acetyl esterase (Jansen *et al.* 1948), B-esterase (Aldridge, 1953) and true and pseudocholinesterase. A-esterase is not inhibited, and in this paper it is shown that it hydrolyses E 600. A consideration of the mechanism of inhibition of enzymes by organophosphorus compounds provides a clue to a possible explanation of this striking difference in behaviour. Jansen *et al.* (1949*a*, *b*) have shown when chymotrypsin is inhibited by di*iso*propyl fluorophosphonate, 1 mol.prop. of hydrofluoric acid is liberated and the di*iso*propyl phosphate radical remains attached to the enzyme. Analogy with this reaction has suggested that the inhibition of other enzymes by such compounds will be by a similar mechanism. This view is substantiated by the fact that when pseudocholinesterase is inhibited by di*iso*propyl fluorophosphonate, phosphorus remains attached to the enzyme (Boursnell & Webb, 1949) and that, for a series of analogues of E 600 of widely varying stabilities to hydrolysis, the more stable the compound is to hydrolysis the less effective it is as an inhibitor (Aldridge & Davison, 1952). The evidence available suggests that the inhibitor behaves as a substrate and is hydrolysed, but that one of the products, the substituted phosphoric acid, remains attached to the enzyme thus preventing its activity. If this be true then all the six enzymes discussed above which can hydrolyse nitrophenyl esters can also hydrolyse E 600, but in only one case (A-esterase) do both products of the hydrolysis pass into the solution leaving the active centre unaltered.

SUMMARY

1. An enzyme has been demonstrated which will hydrolyse diethyl *p*-nitrophenyl phosphate (E 600-esterase).

2. This enzyme is widely distributed in the tissues of the rabbit and the rat and in sera from many different species.

3. Some of the properties of the enzyme in rabbit serum have been examined.

4. By summation experiments, inhibition by diethyl phenyl phosphate, and by the ratio of the activities in different enzyme preparations, it has been shown that E 600-esterase is identical with A-esterase.

5. These enzymes (activities) in the serum of the rabbit, rat and horse have different substrate specificities.

6. The implications of these observations are discussed.

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Studies in Detoxication

45. β-GLUCURONIDASE AND ARYLSULPHATASE IN THE CROP FLUID OF LOCUSTS

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Studies in progress in this laboratory on the detoxication mechanisms of locusts involved an investigation of the occurrence of the enzymes β-glucuronidase and arylsulphatase in these insects. It was soon discovered that a β-glucuronide-decomposing enzyme and an arylsulphatase occurred in the crop liquor of these insects to an appreciable extent.

For the estimation of the β-glucuronidase activity in the crop liquor the easily prepared quinolyl-8-glucuronide was found to be a convenient substrate, whilst arylsulphatase could readily be estimated by means of potassium 2-hydroxy-5-nitrophenylsulphate as suggested by Robinson, Smith, Spencer & Williams (1952).

EXPERIMENTAL

Preparation of locust crop fluid and the enzyme solution

Adult locusts of two species (*Locusta migratoria migratorioides* and *Schistocerca gregaria*) were provided by the Anti-Locust Research Centre, British Museum (Natural History) and were kept on a diet of grass at 35–40° and relative humidity of 70%. The crop fluid of the insects was obtained by picking them up by the thorax and placing the end of a blood pipette to their mouths. On handling in this way, the insects become excited and expel a large drop of clear brownish fluid which then runs into the pipette (for discussion of the production of this drop of fluid see Chauvin, 1941). Volumes up to 0·04 ml. (average 0·02 ml.) could be obtained from each insect during a single manipulation. After a meal of grass the crop liquor is often greenish in colour, cloudy and more dilute than the brown liquor.

For estimation on individual insects the crop fluid was used without dilution, whereas with the pooled fluid from several insects it was diluted with water as needed. Three specimens of pooled crop fluid, on drying at 110°, were shown to contain 10·26, 10·57 and 10·3% of solid matter. The nitrogen content (Kjeldahl) of pooled material was 8·75 mg. N/ml. (two estimations).

Determination of β-glucuronidase activity using p-chlorophenylglucuronide as substrate

In the initial stages of the investigation β-glucuronidase activity was assayed by the method of Spencer & Williams (1951). The crop fluid was diluted with water, usually about 200 times. It was found that the enzyme solutions contained material which absorbed ultraviolet light of wavelength less than 280 mμ., so that it was not always possible to use the 245 mμ. band of the *p*-chlorophenol anion. Estimations were therefore carried out using the 298 mμ. band of this anion. The use of this wavelength was also advantageous because of the high glucuronidase activity of the crop fluid.

Table 1. *β-Glucuronidase activity of brown crop fluid of individual locusts*

(Activity estimated at pH 5·2 with *p*-chlorophenylglucuronide.)

Locust species	Phenol liberated (μg./hr./ml. crop fluid)
Locusta migratoria	3600, 11350, 14000, 14500, 18150
Schistocerca gregaria	9650, 18400, 23500, 30500

All measurements were made at pH 5·2 which, as we found later, was not the optimum pH of the locust enzyme. The results obtained with five specimens of *Locusta migratoria* and four of *Schistocerca gregaria* are given in Table 1 which shows that the crop fluid of these insects may contain from 10 000 to 30 000 chlorophenyl units/ml. of β-glucuronidase at pH 5·2, or about 200–300 units from each drop (0·02 ml.) of fluid obtained by a single manipulation of one of the insects.

Determination of β-glucuronidase with quinolyl-8-glucuronide

Preparation of quinolyl-8-glucuronide. This compound, and some of its metallic salts, was prepared by Brahm (1899), but no rotation or full analysis was quoted for the glucuronide itself. Our method of preparation, however, is new and relatively simple. A total of 18 g. of 8-hydroxyquinoline were fed by stomach tube, in water, to six rabbits. The 24 hr. urine (750 ml.), after filtration through glass wool, was brought to pH 4 with glacial acetic acid (about 3 ml.) and left at 0° for 2 days. The β-8-quinolylglucuronide separated slowly as large greenish crystals (12 g.) which were filtered and then recrystallized several times (charcoal) from hot water. The second 24 hr. urine, by the same procedure, yielded a further 1 g. of crude glucuronide. The *trihydrate* formed pale yellow-green prisms of m.p. 153° (decomp.); $[\alpha]_D^{20°} - 75° \pm 2·5°$ in water (*c*, 0·1). (Found: C, 47·4; H, 5·6; N, 3·7. $C_{15}H_{15}O_7N.3H_2O$ requires C, 48·0; H, 5·6; N, 3·7%.) It was sparingly soluble in water, ethanol and ether, but more soluble in boiling water.

The colour reaction between 8-hydroxyquinoline and diazotized di-o-anisidine

Conditions of colour development. When a solution of Brentamine Fast Blue B salt (referred to later as BFB) (Imperial Chemical Industries, Ltd.) is added to 8-hydroxy-

quinoline a cherry-red colour develops which is proportional to the amount of phenol used. Quinolyl-8-glucuronide gives no colour with BFB. The colour obtained is stable for 20 min. after reaching its maximum, between the pH limits of 4 and 6·5. Above pH 6·5 the colour tends to precipitate, whereas below pH 4 it develops extremely slowly. The limits of stability of the colour are therefore within the pH range of maximum activity of β-glucuronidase. The solution of BFB was therefore made up in a solution of 0·01 M-potassium acid saccharate in 0·3 M-acetate buffer at pH 4·5, the saccharate serving to inhibit the β-glucuronidase, and the acetate to provide a pH at which the colour has a maximum stability. A 0·1 % solution of BFB was found to be the most suitable and economical. Using these solutions the colour attained its maximum in 5 min. at 37°, or in 15 min. at room temperature.

The standard curve was prepared as follows. A solution of 8-hydroxyquinoline (3 ml., 4–30 μg.) in 0·3 M-acetate buffer at pH 4·5 was treated with 1 ml. of 0·1 % BFB. The solution was allowed to stand 15 min. at room temperature and then the colour measured in the Spekker absorptiometer using an Ilford no. 603 blue-green filter and 0·5 ml. cells. The standard curve obtained was a straight line.

Method of assay

Enzyme solution. In most cases, 0·1 ml. of crop fluid diluted to 200 ml. with distilled water contained enough enzyme for incubation periods up to 1 hr.

Substrate solution. Quinolyl-8-glucuronide is very sparingly soluble in water (about 0·003 M), but fortunately the optimum substrate concentration (see below) is about 0·0003 M and a 0·001 M solution of the glucuronide is easily obtained. The stock substrate solution was therefore a 0·001 M solution of the glucuronide in water. This solution does not deteriorate in 4–5 days.

BFB solution. This consisted of a 0·1 % solution of BFB in a solution of 0·01 M-potassium acid saccharate in 0·3 M-acetate buffer of pH 4·5. This solution has to be freshly made just before use since it deteriorates in a few hours.

The final method of assay of β-glucuronidase in the crop fluid was therefore as follows. To each of two tubes was added 1 ml. of 0·001 M-substrate solution, 1 ml. M-acetate buffer of pH 4·5 and 1 ml. of enzyme solution. These tubes were then incubated at 37°, together with a control tube containing 1 ml. substrate and 1 ml. acetate buffer. After the desired period of incubation (usually 1 hr.), 1 ml. of the BFB solution was added to all three tubes and 1 ml. of the enzyme solution to the control tube. After 5 min. (at 37°) the colour of the test solutions was measured in the Spekker absorptiometer (filter, Ilford no. 603 and 0·5 ml. cells) with the control solution in the blank cell of the instrument. The amount of 8-hydroxyquinoline liberated was then read off from the standard curve (see Tables 2 and 3 for results). This method was not applicable to the assay of β-glucuronidase of animal tissues (e.g. rat liver) since the tissue extracts interfered considerably with the development of colour.

Table 2. *β-Glucuronidase content of fluids of* Locusta migratoria

(Activity estimated with quinolyl-8-glucuronide at pH 4·5.)

Fluid	8-Hydroxyquinoline liberated (μg./hr./ml.)
A pooled sample of brown crop fluid	64 000
Green crop fluid from individual insects	3850, 5500, 8000, 9700, 14 000
Pooled crop fluid from 20 hoppers (5th instar)	4200
Pooled haemolymph* from five adults	300
Ecdysial fluid†	<200 (5 specimens)

* To obtain haemolymph, the insect was punctured in the thorax and gently squeezed, and the drop of pale yellow-green fluid (about 0·05 ml.) which appeared was collected in a blood pipette.

† When the insect is about to moult it hangs upside down and moves its abdomen. When found in this position, one section of the abdomen was removed with a sharp scalpel and then the old skin came away easily. The unbroken new skin could be seen underneath. The ecdysial fluid is found between the old and new skins and resembles haemolymph. It was collected by means of a blood pipette and as much as 0·2 ml. could be obtained from one locust.

Optimum pH

For the pooled enzyme solution this was determined as above, except that the pH of the acetate buffer was varied from pH 3·8 to 5·7. A single optimum was found at pH 4·5 (see Fig. 1). Under standard conditions the amount of 8-hydroxyquinoline liberated by a constant amount of a given specimen of crop liquor was found to be proportional to the time of incubation, up to 4 hr. The optimum substrate concentration was determined at pH 4·5 and 37° as above, using

Table 3. *β-Glucuronidase activity of locust* (Locusta migratoria) *crop fluid at different stages of development*

Age of locust (days)	Stage of development (instar)	Activity of crop fluid of individual insects at pH 4·5 (μg. 8-hydroxyquinoline liberated/hr./ml.) Average	Range	No. of individual insects tested
6	1st	5 200	590–12 200	9
8	or 2nd?	18 200	9 700–24 000	6
13	3rd	6 890	3 150–10 600	5
17		11 400	8 300–14 800	6
21		14 300	9 700–16 000	6
23	4th	7 020	2 500–12 000	5
26		16 300	14 800–18 000	6
28	5th	16 300	13 000–17 750	8
34		20 560	16 000–24 000	5

substrate concentrations varying from 0·1 to 10×10^{-4} M and incubating for 1 hr. Maximum activity under these conditions was attained at a concentration of 3×10^{-4} M. Beyond this value there was no increase in activity and no inhibition by excess substrate.

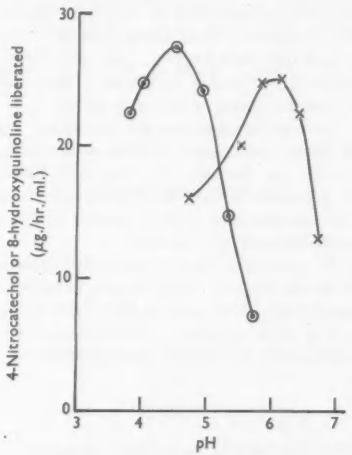

Fig. 1. pH-Activity curves for the β-glucuronidase (⊙—⊙) and arylsulphatase (×—×) of crude locust crop fluid (*Locusta migratoria*).

Inhibition by saccharate

D-Glucosaccharic acid has been shown to be a specific inhibitor of β-glucuronidase from mammalian tissues (Karunairatnam & Levvy, 1949). The enzyme of locust crop fluid is also inhibited by this substance. To study this effect potassium acid saccharate was incorporated in the acetate buffer. The procedure described above was used. A 50% inhibition of the enzyme acting on 0·0003 M-quinolyl-8-glucuronide at pH 4·5 and 37° was noted when the saccharate concentration reached 0·0004 M, whilst complete inhibition was observed at 0·01 M. These results are similar to those found by Karunairatnam & Levvy (1951) for mouse-liver β-glucuronidase which is completely inhibited by 0·01 M-saccharate when acting on 0·00125 M-phenolphthalein glucuronide at pH 4·5 in acetate buffer. This suggests that the locust enzyme is a β-glucuronidase. (Recent work by Levvy (1952) suggests that the inhibition by saccharate solutions is probably due to the presence of saccharo-1:4-lactone. To ensure the presence of this lactone in saccharate solutions they should be boiled before use.)

Detection of glucuronic acid. Karunairatnam & Levvy (1951) have pointed out that the detection of a phenol as a result of the action of an enzyme preparation supposed to contain β-glucuronidase on a phenolic glucuronide is not sufficient to characterize the enzyme as a β-glucuronidase. The detection of the glucuronic acid is also necessary (cf. Levvy, 1948). Saturated aqueous solutions (1 ml.) of several glucuronides were therefore incubated at 37° for 18 hr. with 0·2 ml. of pooled locust crop liquor, and 0·2 ml. of each solution was then separated chromatographically on paper in *n*-butanol:acetic acid:water (cf. Partridge, 1948), along with authentic samples of D-xylose, glucuronic acid and glucurone. Quinolyl-8-glucuronide, *o*- and *m*-aminophenylglucuronides and *p*-iodophenylglucuronide were used and in each case glucuronic acid, but not glucurone or xylose, was demonstrated using a naphthoresorcinol spray (Partridge, 1948). Free *o*- and *m*-aminophenols were revealed by the salicylaldehyde reagent (Robinson, Smith & Williams, 1951*a*) and 8-hydroxyquinoline by its fluorescence in ultraviolet light. Crop liquor, at the concentration used, gave no interfering spots.

These results, therefore, show that locust crop liquor has an enzyme with the properties of a true β-glucuronidase.

Arylsulphatase activity

The arylsulphatase activity of locust crop liquor was determined by one of the methods of Robinson *et al.* (1952). This method depends on the release from potassium 2-hydroxy-5-nitrophenylsulphate of 4-nitrocatechol which gives a red colour with alkali.

It was assumed that the optimum substrate concentration of the crop liquor sulphatase would be similar to that of takadiastase, i.e. 0·0025 M (Robinson *et al.* 1952), and the enzyme solution used was prepared by diluting locust crop liquor roughly 25 to 50 times.

With individual locusts a measured volume (approx. 0·02 ml.) of the crop fluid was added to 2 ml. of buffer-substrate solution (0·0025 M-potassium 2-hydroxy-5-nitrophenylsulphate in 0·5 M-acetate buffer of pH 5·9). This solution and a control buffer-substrate solution (2 ml.) were incubated at 37° for 1 hr.

The results (Table 4) show that locust crop liquor contains appreciable amounts of arylsulphatase (approx. 1000 4-nitrocatechol units/ml. or 100–120 units/mg. N) comparable with the takadiastase (800–900 units/g. or 200 units/mg. N) used in earlier experiments (Robinson *et al.* 1952).

Table 4. *The arylsulphatase activity of the crop fluid and haemolymph of locusts*

Animal	Source	4-Nitrocatechol liberated (μg./ml./hr.)
Locusts		
Locusta migratoria	Brown crop fluid	850, 900, 960, 1000, 1035*
	Haemolymph	67·5, 137, 187, 165*
Schistocerca gregaria	Brown crop fluid	1300, 1130*
Humans	Saliva (5 specimens)	0
Goat	Saliva (1 specimen)	0
Frog hoppers	'Cuckoo spit'	0

* Specimens from different insects.

The haemolymph of these insects was also tested and was found to possess about one-tenth of the activity of the crop liquor. Human and goat saliva contained no arylsulphatase activity nor did the froth ('cuckoo spit') of frog hoppers (see Table 4).

Optimum pH. For the locust enzyme this was determined essentially as above, varying the pH of the buffer. It was found to be in the region of pH 6 (see Fig. 1), and is thus similar to that of takadiastase (Robinson *et al.* 1952).

Inhibitors of locust arylsulphatase

Some of the inhibitors which inhibited the arylsulphatase activity of takadiastase (Robinson *et al.* 1952) were also tested against the locust enzyme. For this purpose 2 ml. of 0·04 M inhibitor solution in M-acetate buffer at pH 5·9 were incubated with 1 ml. of 0·1 M substrate solution in M-acetate buffer at pH 5·9 and 1 ml. of the diluted crop fluid, together with the appropriate control without the enzyme. The colour was developed by adding 1 ml. of 10% (w/v) NaOH to the test solutions and 1 ml. each of NaOH and of enzyme solution to the control. The inhibition found was very similar in extent to that of takadiastase (see Robinson *et al.* 1952). With 0·02 M-K_2SO_3, NH_2OH, HCl and KCN, the inhibition was 87, 80 and 100% respectively. Dzialoszynski (1947) has reported that urea inhibits the arylsulphatase of 'Clarase'. We also tested urea against the locust enzyme and found that a 0·02 M solution caused a 24% inhibition of the enzyme.

An attempt has been made to apply the above method to partly purified extracts of animal tissues (Robinson, Smith & Williams, 1951*b*). Dr Roy of Edinburgh University (in a private communication) has carefully applied the method to tissues and informs us that the method will probably not be of any value for the assay of animal sulphatase, as fairly highly purified preparations must be used to ensure quantitative recovery of the liberated nitrocatechol, and also the kinetics of the reaction appear to be complex. He has come to the conclusion that the animal and mould enzymes have very different properties (see Roy, 1953).

DISCUSSION

The crop liquor of locusts has been shown to contain a number of enzymes which are obviously related to digestion, for Chauvin (1941) reports that it contains a dextrinase, an amylase and a sucrase (see also Uvarov, 1948). It is not clear whether the sulphatase and glucuronidase we have found in crop liquor is secreted by the locust or is of microbiological origin. Dr A. E. Oxford has examined the crop fluid for us and found no large Protozoa, but some medium-sized micro-organisms more or less similar to those found, by Karunairatnam & Levvy (1951), to carry most of the glucuronide-decomposing activity of sheep rumen contents. This may be associated with the digestion of polysaccharides containing β-glucuronoside links as has been suggested by Marsh, Alexander & Levvy (1952) for a number of domestic animals, and the relatively high activity may be correlated with the reported high rate of digestion in locusts (Uvarov, 1948). A digestive role for these enzymes seems to be supported by the slight activity of haemolymph and ecdysial fluid (Table 2) and by the observation that the glucuronidase activity of crop fluid tends to be less at the beginning of an instar (Table 3) when the insects have not been feeding. Normally, the locust stops feeding on the day before moulting and does not feed readily again till the day after.

It is interesting to note that the locust enzyme has the same optimum pH (4·5) with quinolyl-8-glucuronide as Sarkar & Sumner (1950) and Talalay, Fishman & Huggins (1946) have found for glucuronidase preparations of animal origin acting on phenolphthalein glucuronide.

The pH optimum of locust arylsulphatase, about pH 6, was also similar to that found in takadiastase (Robinson *et al.* 1952) and rat liver (Robinson *et al.* 1951*b*), but there appears at the moment to be no obvious function for this enzyme in locusts.

SUMMARY

1. The crop fluid of two species of locusts has been proved to contain appreciable amounts of β-glucuronidase and an arylsulphatase.

2. The β-glucuronidase was estimated with quinolyl-8-glucuronide as substrate, the liberated 8-hydroxyquinoline being estimated by coupling under specified conditions with Brentamine Fast Blue B salt (diazotized *o*-anisidine).

3. The β-glucuronidase of the crude crop liquor showed a single pH optimum at 4·5 and was present in all stages of development from the first instar to the full adult.

4. The formation of glucuronic acid by the action of the crop liquor on a number of β-glucuronides was proved by paper chromatography.

5. Arylsulphatase was detected by the release of 4-nitrocatechol from potassium 2-hydroxy-5-nitrophenylsulphate. With this substrate the enzyme showed an optimum at about pH 6.

6. Arylsulphatase activity was not found in human or goat salivas.

7. The results are discussed and the suggestion made that β-glucuronidase may be connected with digestion in locusts.

We are grateful to the Anti-Locust Research Centre (British Museum) for supplies of locusts, to Imperial Chemical Industries, Ltd., for a gift of Brentamine Fast Blue B salt, to Prof. E. C. Amoroso for a sample of goat saliva, to Dr A. E. Oxford for microscopic examination of locust crop liquor and to Dr G. A. Levvy for information prior to publication.

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Studies in Detoxication

46. THE METABOLISM OF ALIPHATIC ALCOHOLS. THE GLUCURONIC ACID CONJUGATION OF ACYCLIC ALIPHATIC ALCOHOLS

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A large number of aliphatic alcohols are used industrially as solvents and as starting materials for the manufacture of other chemicals such as esters and ethers. Those most commonly used are the monohydric and dihydric alcohols, and glycerol. According to von Oettingen (1943) those containing more than three hydroxyl groups are of no industrial toxicological importance. Aliphatic alcohols, other than ethanol, also occur in small amounts in wines and liquors, but their nature and amounts are not accurately known. In analysis, these alcohols are lumped together as 'higher alcohols' or fusel oil. Rum, brandy and whisky may contain 100–400 mg./100 ml. of higher alcohols, the amounts varying according to the 'mash' used, the yeast culture employed and the conditions of fermentation. *n*-Propanol, *iso*propanol, *n*-butanol, *iso*butanol, *n*-, active and *iso*-amyl alcohols have been reported in fusel oil (Kirk & Othmer, 1947). In addition to these alcohols, heptyl alcohol has been reported in a 25-year-old brandy (Ordinneau, 1886; cf. Herstein & Jacobs, 1948), and Hewitt (1928) states that alcohols as high as octyl and nonyl occur in some wines and spirits. The bouquet or flavour of wines is in part due to the presence of esters of these alcohols. The amyl alcohols appear to arise by fermentation of leucine and isoleucine (Herstein & Jacobs, 1948). Appreciable quantities of higher alcohols are therefore being constantly consumed by human beings. Methanol up to 0·36 % may occur in the cheap brandies (marc brandy); this usually arises as the result of the fermentation of pectin.

Most of the known data on the toxicity and metabolism of the aliphatic alcohols have been assembled by von Oettingen (1943), and it is clear from his publication that our knowledge of the metabolism of these compounds is but fragmentary. In general, however, it is known that aliphatic alcohols are oxidized *in vivo*, the primary alcohols being initially oxidized to aldehydes and the secondary to ketones (see Williams, 1947). Some of the more volatile alcohols are undoubtedly eliminated to some extent in the unchanged state by the lungs. The tertiary alcohols, *tert.*-butyl and amyl are known to be partly excreted in the urine as glucuronides. Our knowledge of the fate of methanol and ethanol is fairly extensive, and a valuable study of the metabolism of seven out of the eight possible isomers of amyl alcohol has been made by Haggard, Miller & Greenberg (1945). The latter workers showed quite clearly that the rate of the metabolism of the amyl alcohols in the rat was in the order primary > secondary > tertiary.

In most of the studies on alcohols few workers have paid any attention to whether these alcohols give rise to conjugated glucuronic acids. Neubauer (1901), from qualitative observations on dogs and rabbits, concluded that a number of alcohols were excreted as glucuronides, including ethanol but not methanol. Deichmann & Thomas (1943), however,

found neither methanol (2·3 ml./kg.) nor ethanol (3·5 ml./kg.) to affect the glucuronic acid output of the rabbits. Thierfelder & Mering (1885) showed, by isolation of the glucuronides, that *tert.*-butyl and amyl alcohols were conjugated by rabbits.

It is also possible that aliphatic alcohols conjugate with sulphuric acid. According to Pringsheim (1908) ethanol is excreted to a slight extent as an ethereal sulphate. However, Anderton, Smith & Williams (1948) found no increase in the ethereal sulphate output of rabbits receiving ethanol, and Thierfelder & Mering (1885) have reported similarly for *tert.*-butyl alcohol. In the present work three alcohols, *tert.*-amyl alcohol, pentan-2-ol and pentan-3-ol, were tested for their effect on the ethereal sulphate output of rabbits, but in no case was ethereal sulphate formation detected.

The object of the present work was to find out to what extent various monohydric aliphatic alcohols conjugate with glucuronic acid in the rabbit, to relate the findings to the structure and other properties of the alcohol and to make any relevant observation concerning other paths of metabolism of the alcohol.

EXPERIMENTAL

Alcohols

Most of the alcohols used were purchased and were of laboratory reagent quality. All alcohols were redistilled and boiling points checked before use. The *n*-butyl, *sec.*-butyl and *iso*butyl alcohols were purified through their respective hydrogen phthalates and *tert.*-butyl alcohol through its *p*-nitrobenzoate before finally distilling. *iso*Butylmethylcarbinol, 2-ethylbutan-1-ol, *n*-hexyl alcohol, di*iso*propylcarbinol and 2-ethylhexan-1-ol were British Drug Houses Ltd. technical grade alcohols and were fractionated before use. All boiling points agreed with those given by Huntress & Mulliken (1946) and, where necessary, solid derivatives were prepared and melting points checked.

Heptan-2-ol was prepared by reduction of methyl *n*-amyl ketone (see Blatt, 1943). All alcohols used containing an asymmetric carbon atom were DL forms (2-ethylbutanol, 2-ethylhexanol, butan-2-ol, pentan-2-ol, hexan-2-ol, 4-methylpentan-2-ol, heptan-2-ol, heptan-3-ol and octan-2-ol), and this was checked polarimetrically.

Quantitative estimations

Large chinchilla rabbits of about 3 kg. weight were used and kept on a constant diet of 75 g. rat cubes/day: The animals were kept on this diet for at least 1 week before administration of an alcohol in order to obtain as steady a value as possible for the normal output of glucuronic acid. The excretion of glucuronic acid was determined daily by the method of Hanson, Mills & Williams (1944). The alcohols were administered with water by stomach tube and the glucuronic acid output for each alcohol was determined simultaneously on three animals. The dose of alcohol in most cases was 25 m-moles/rabbit. The results are given in Tables 1–4. In most instances the excretion of extra glucuronic acid after feeding these alcohols was complete within 24 hr. Only in the cases of *iso*propanol, *n*-decanol, di*iso*propylcarbinol, *tert.*-amyl alcohol, 2-ethylhexanol and 3-heptanol did extra glucuronic acid appear in the urine on the second day after dosing. Ethereal sulphates were determined by the method of Sperber (1948).

Qualitative experiments

Melting points are uncorrected.

Isolation of the glucuronides. Only in a few cases was it possible to isolate the free glucuronides of the aliphatic alcohols. They usually formed non-crystalline water-soluble gums. In some instances, however, crystalline salts with *p*-toluidine and benzylamine were obtained and occasionally a crystalline potassium salt was isolated. In almost every case, however, the glucuronide could be obtained as a crystalline triacetyl methyl ester. All these aliphatic esters were highly soluble in ethanol except the ethyl derivative.

In most cases the crude glucuronides were isolated from the urine by extraction with ether or mixtures of ether and ethanol, but in some instances isolation through the basic lead salt, as described by Kamil, Smith & Williams (1951) for the substituted phenylglucuronides was successful. Many aliphatic glucuronides are, however, not completely precipitated by basic lead acetate even when the latter is made highly alkaline with NH_4OH. These glucuronides could be readily extracted either by continuous extraction of the urine with ether or by extraction in a separating funnel with ether/ethanol mixtures. The urine of rabbits fed

Table 1. *The glucuronic acid conjugation of normal aliphatic alcohols in the rabbit*

Alcohol			Extra glucuronic acid excreted	
Name (normal)	Formula	Dose (m-moles/3 kg.)	(% of dose)	Average
Methyl	CH_3OH	75	0, 0, 0	0
Ethyl	C_2H_5OH	50	0·58, 0·32, 0·56	0·5
n-Propyl	C_3H_7OH	40	0·8, 1·1, 0·8	0·9
n-Butyl	C_4H_9OH	16	1·1, 1·0, 3·4	1·8
n-Amyl	$C_5H_{11}OH$	25	6·5, 7·4, 6·1	6·7
n-Hexyl	$C_6H_{13}OH$	25	10·0, 8·4, 12·4	10·3
n-Heptyl	$C_7H_{15}OH$	25	5·5, 4·7, 5·3	5·3
n-Octyl	$C_8H_{17}OH$	25	12·5, 7·8, 8·2	9·5
n-Nonyl	$C_9H_{19}OH$	25	4·6, 3·3, 4·5	4·1
n-Decyl*	$C_{10}H_{21}OH$	25	3·5, 3·6*	3·5
n-Octadecyl*	$C_{18}H_{37}OH$	25	5·1, 10·1*	—

* Absorption of the alcohol incomplete and irregular, and the alcohol could be isolated in quantity from the faeces.

Table 2. *The glucuronic acid conjugation of arborescent primary alcohols in the rabbit*

Alcohol*	Formula	Extra glucuronic acid excreted: % of dose	Extra glucuronic acid excreted: Average
Branching at β-carbon			
*iso*Butanol (β:β-dimethylethanol)	$(CH_3)_2CH.CH_2OH$	2·7, 6·3, 4·1	4·4
2-Methylbutan-1-ol (β-methyl-β-ethylethanol)	$C_2H_5.CH(CH_3)CH_2OH$	9·1, 10·4, 9·2	9·6
2-Ethylbutan-1-ol (β:β-diethylethanol)	$(C_2H_5)_2CH.CH_2OH$	49, 36, 34	40†
2-Ethylhexan-1-ol (β-ethyl-β-*n*-butylethanol)	$n\text{-}C_4H_7.CH(C_2H_5)CH_2OH$	89·6, 88·1, 83·0	86·9†
Branching at γ-carbon			
3-Methylbutan-1-ol (primary *iso*amyl)	$(CH_3)_2CH.CH_2CH_2OH$	11·0, 7·7, 8·4	9·0

* Dose: 25 m-moles/3 kg. rabbit.
† Mainly reducing glucuronide of corresponding fatty acid (see text).

Table 3. *The glucuronic acid conjugation of some acyclic aliphatic alcohols in the rabbit*

Alcohol*	Formula	Extra glucuronic acid excreted: % of dose	Extra glucuronic acid excreted: Average
Propan-2-ol (*iso*propanol)	$CH_3.CHOH.CH_3$	8·9, 13·5, 8·2	10·2
Butan-2-ol (*sec.*-butanol)	$C_2H_5.CHOH.CH_3$	15·3, 16·4, 11·7	14·4
Pentan-2-ol (methyl-*n*-propylcarbinol)	$n\text{-}C_3H_7.CHOH.CH_3$	47·3, 45·4, 41·7	44·8
Pentan-3-ol (diethylcarbinol)	$C_2H_5.CHOH.C_2H_5$	40·1, 42·1, 35·3	39·2
Hexan-2-ol (methyl-*n*-butylcarbinol)	$n\text{-}C_4H_9CHOH.CH_3$	48·3, 60·9, 53·8	54·3
4-Methylpentan-2-ol (methyl*iso*butylcarbinol)	$iso\text{-}C_4H_9.CHOH.CH_3$	36·5, 37·3, 27·8	33·7
Heptan-2-ol (methyl-*n*-amylcarbinol)	$n\text{-}C_5H_{11}.CHOH.CH_3$	57·7, 57·5, 48·8	54·6
Heptan-3-ol (ethyl-*n*-butylcarbinol)	$n\text{-}C_4H_9.CHOH.C_2H_5$	58·6, 63·9, 63·2	61·9
Heptan-4-ol (di-*n*-propylcarbinol)	$n\text{-}C_3H_7.CHOH.n\text{-}C_3H_7$	69·9, 69·5, 62·9	67·4
2:4-Dimethylpentan-3-ol (di*iso*propylcarbinol)	$iso\text{-}C_3H_7.CHOH.iso\text{-}C_3H_7$	60·4, 64·6, 75·2	66·4
Octan-2-ol (methyl-*n*-hexylcarbinol)	$n\text{-}C_6H_{13}.CHOH.CH_3$	14·5, 16·6 —	15·5

* Dose: 25 m-moles/3 kg. of rabbit, except in the case of *iso*propanol where the dose was 50 m-moles/kg.

Table 4. *The glucuronic acid conjugation of acyclic aliphatic tertiary alcohols in the rabbit*

Alcohol	Formula	Dose (m-moles/3 kg.)	Extra glucuronic acid excreted: % of dose	Extra glucuronic acid excreted: Average
Trimethylcarbinol (*tert.*-butyl)	$(CH_3)_3COH$	12	27·2, 23·1, 22·9	24·4
Dimethylethylcarbinol (*tert.*-amyl)	$C_2H_5(CH_3)_2COH$	15	58·5, 54·3, 60·8	57·5
Dimethyl-*n*-propylcarbinol (*tert.*-hexyl)	$C_3H_7(CH_3)_2COH$	25	61·5, 50·2, 58·9	56·7

Table 5. *The glucuronic acid conjugation of primary, secondary and tertiary alcohols*

Alcohol	Conjugation (as % of dose)			
	Primary		Secondary	Tertiary
Propyl		0·9	10·2	—
Butyl	Normal	1·8	14·4	24·4
	iso	4·4		
Amyl	Normal	6·7	Pentan-2-ol 44·8	57·5
	iso	9·0	Pentan-3-ol 39·2	
	2-Methylbutanol	9·6		
Hexyl	Normal	10·3	Hexan-2-ol 54·3	56·7

with the alcohols was collected for 24 hr., saturated with solid $(NH_4)_2SO_4$ and acidified to pH 1–2 with conc. HCl. If extraction is carried out in a separating funnel the acidified urine is shaken with 0·5–1 vol. of ether, or 1:2, 1:3 or 1:4 ethanol/ether mixtures according to the solubility of the glucuronide in these solvents as indicated by preliminary tests. The extraction is repeated at least three times. If the extraction is carried out in a continuous extractor, it is made with ether and allowed to proceed for 16 hr. The extract is then dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure at 40–50°.

The crude glucuronide gums obtained by these methods, after attempts were made to crystallize them or form crystalline salts, were methylated in methanol with ethereal diazomethane and then acetylated with pyridine and acetic anhydride to convert them to triacetyl methyl esters (cf. Kamil *et al.* 1951). The crude triacetyl methyl esters were recrystallized from aqueous ethanol and finally from *n*-hexane.

The optical rotations of the triacetyl methyl esters are tabulated (see Table 6).

Normal primary alcohols

It is clear from Table 1 that the conjugation of these alcohols is very low. Attempts, however, were made to isolate the glucuronides of methyl, ethyl, *n*-amyl, *n*-hexyl and *n*-octyl alcohols. Success was only achieved with the methyl, ethyl and *n*-hexyl derivatives. The isolation of methyl and ethyl glucuronides and observations on the metabolism of methanol and ethanol will be published later. To obtain the glucuronide gum of *n*-hexyl alcohol, the 24 hr. urine of three rabbits which had each received 3 ml. of *n*-hexyl alcohol by stomach tube was extracted as described above with 1:4 ethanol/ether. From the gum there was prepared *triacetyl β-n-hexyl-D-glucuronide methyl ester* which slowly crystallized during 2 months from aqueous ethanol as long, white, silky needles, m.p. 70–71°. (Found: C, 55·1; H, 7·3. $C_{19}H_{30}O_{10}$ requires C, 54·5; H, 7·2%.) (Yield, 0·35 g.)

Arborescent primary alcohols

iso*Butanol.* Gray, Adams & Hauptmann (1950) have suggested from studies using [*carboxy*-^{14}C] and [*methyl*-^{14}C]-*iso*butyric acid that, in rats, *iso*butyric acid is decarboxylated and may give rise to acetone. *iso*Butanol could therefore give rise to *iso*butyraldehyde and *iso*butyric acid by oxidation, and possibly acetone via *iso*butyric acid. A rabbit was therefore given 6 ml. of *iso*butanol by stomach tube and placed in the tank described by Parke & Williams (1950). The expired air was drawn through Brady's reagent (2:4-dinitrophenylhydrazine in HCl) for 6 hr. No hydrazone was formed and it was concluded that no volatile aldehyde or ketone was eliminated via the lungs. The urine from rabbits receiving *iso*butanol was non-reducing and contained no aldehyde or ketone when tested with Brady's reagent. The glucuronic acid conjugation of this alcohol was low but significant (see Table 2).

DL-2-*Methylbutanol and* iso*amyl alcohol.* These gave urines which did not reduce Benedict's reagent but gave positive naphthoresorcinol tests. The urines from both alcohols did not contain aldehydes or ketones. Both these alcohols yielded about 9–10% of non-reducing glucuronide as determined by the naphthoresorcinol method (see Table 2). In the case of *iso*amyl alcohol, the glucuronide was isolated. The 24 hr. urines from six rabbits which had received a total of 14 g. of *iso*amyl alcohol were worked up by the basic lead acetate method and yielded 0·5 g. of glucuronide gum. This yielded 0·1 g. of *triacetyl β-iso*amyl-D-*glucuronide methyl ester* which formed colourless needles, m.p. 96°, after recrystallization from acetic acid and finally *n*-hexane. (Found: C, 54·0; H, 6·7. $C_{18}H_{28}O_{10}$ requires C, 53·5; H, 7·0%.)

Our studies on 2-ethylbutanol and 2-ethylhexanol are described in the succeeding paper (Kamil, Smith & Williams, 1953).

Secondary alcohols

iso*Propanol.* Neubauer (1901) has referred to a severe case of diabetes studied by Mayer (1899) in which there was a considerable acetone excretion and a large quantity of conjugated glucuronic acid; Neubauer suggested that this glucuronide may have been *iso*propylglucuronide. *iso*-Propanol has also been found in the blood, milk and rumen contents of cows suffering from acetonaemia, and Robertson, Thin & Sterling (1950) suggest that it may be a precursor or a metabolite of acetone. The conversion of *iso*propanol to acetone *in vivo* is well known (cf. Neymark, 1938). Neubauer (1901) has shown by qualitative tests that *iso*propanol increases the output of glucuronic acid in dogs and rabbits. In the present work the conjugation of *iso*propanol with glucuronic acid has been confirmed and the glucuronide isolated.

The expired air of a rabbit which had received 6 ml. of *iso*propanol was drawn through Brady's reagent for 6 hr. after feeding the alcohol. There were isolated 94 mg. (0·5% of dose) of acetone 2:4-dinitrophenylhydrazone, m.p. 124° and mixed m.p. 122° after recrystallization. (Found: N, 23·1%. Calc. for $C_9H_{10}N_4O_4$: N, 23·5%.)

The glucuronide of *iso*propanol was isolated from the 24 hr. urine of seven rabbits which had each received 5 ml. of the alcohol. The acidified $(NH_4)_2SO_4$-saturated urine was extracted with 1:2 ethanol/ether and the extract gave a

gum on evaporation which eventually yielded 0·7 g. of crude *triacetyl β-isopropyl-D-glucuronide methyl ester*. This formed colourless needles, m.p. 140°, after recrystallization from aqueous ethanol and finally *n*-hexane. (Found: C, 51·5; H, 6·4. $C_{16}H_{24}O_{10}$ requires C, 51·1; H, 6·4%.)

DL-*Butan-2-ol*. No studies appear to have been made on this alcohol. In rabbits it appears to form a conjugated glucuronide to the extent of about 14–15% (Table 3). It is also oxidized to ethyl methyl ketone for, in an experiment where the expired air of a rabbit, which had been fed 10 ml. of the alcohol, was drawn through Brady's reagent, we isolated 207 mg. (0·75% of the dose) of ethyl methyl ketone 2:4-dinitrophenylhydrazone, m.p. and mixed m.p. 110°. (Found: C, 47·2; H, 4·6; N, 22·6%. Calc. for $C_{10}H_{12}N_4O_4$: C, 47·6; H, 4·8; N, 22·2%.)

sec.-*Butylglucuronide*. This was isolated from a 24 hr. urine by the basic lead acetate method after feeding three rabbits with 8 ml. each of the alcohol. The glucuronide was a gum (3 g.) which yielded crystalline *triacetyl β-sec.-butyl-D-glucuronide methyl ester* as needles, m.p. 107°, from aqueous ethanol and then *n*-hexane. (Found: C, 52·6; H, 6·7. $C_{17}H_{26}O_{10}$ requires C, 52·3; H, 6·7%.)

Secondary amyl alcohols

Haggard *et al.* (1945) have shown that the secondary pentanols are converted in rats into the corresponding ketones, 38–54% of the dose appearing as ketones in the expired air and urine. Small amounts of the alcohols (0·5–11% of the dose) are also eliminated unchanged by these two routes. We have now found that in rabbits some 40% of pentan-2- and -3-ols are excreted as glucuronides (Table 3). Rabbits which received 0·73 g./kg. of these alcohols fell asleep for several hours. Glucuronides, but no ketones, were isolated from their urines.

Pentan-2-ol. The glucuronide of this alcohol was isolated by the basic lead acetate method. Three rabbits, which had collectively received 6·7 g. of the alcohol, yielded 4·55 g. of purified glucuronide gum in 24 hr. Half of this gum yielded 1·72 g. of *triacetyl β-pentyl-2-D-glucuronide methyl ester*, m.p. 90°. (Found: C, 53·6; H, 7·0. $C_{18}H_{28}O_{10}$ requires C, 53·5; H, 7·0%.) The rest of the dried gum (2 g.) in dry ether with *p*-toluidine (2 g. in ether) yielded the crystalline *p*-toluidine salt of *pentyl-2-glucuronide*, m.p. 132° after recrystallization from ethanol and $[\alpha]_D - 30·3°$ in water (*c*, 1). (Found: C, 57·8; H, 7·8; N, 3·9. $C_{18}H_{29}O_7N$ requires C, 58·2; H, 7·9; N, 3·8%.)

This alcohol did not increase the ethereal sulphate output of the rabbits.

Pentan-3-ol. In this case the glucuronide gum was isolated by extraction of the acidified $(NH_4)_2SO_4$-saturated urine with 1:3 ethanol/ether, and 6·7 g. of the alcohol yielded 7·31 g. of glucuronide gum. From 5·3 g. of the gum there were obtained 4·95 g. of *triacetyl β-pentyl-3-D-glucuronide methyl ester*, m.p. 76°. (Found: C, 53·8; H, 6·9%.)

This alcohol did not increase the ethereal sulphate output of rabbits.

Secondary hexyl alcohols

Hexan-2-ol. The urine was non-reducing to Benedict solution and gave a positive Rothera test and a faint turbidity with Brady's reagent overnight. Thus a small amount of a methyl ketone was being excreted. The naphthoresorcinol test was intense and the glucuronide was extracted (continuous) from the acidified urine with ether. The glucuronide gum (5 g.) yielded *triacetyl β-hexyl-2-D-glucuronide methyl ester* as needles, m.p. 80–85°, from *n*-hexane. (Found: C, 54·6; H, 7·4. $C_{19}H_{30}O_{10}$ requires C, 54·5; H, 7·2%.)

4-Methylpentan-2-ol. The urine from rabbits receiving this alcohol gave a non-reducing urine giving a faint Rothera test and a faint turbidity with Brady's reagent. A small amount of a methyl ketone (presumably *iso*butyl methyl ketone) was thus appearing in the urine. The 24 hr. urine from four rabbits which had each received 3 ml. yielded by extraction with 1:4 ethanol/ether a glucuronide gum which was characterized as *triacetyl β-4-methylpentyl-2-D-glucuronide methyl ester*, m.p. 119° (yield, 5·4 g.). (Found: C, 53·7; H, 7·4%.)

Secondary heptyl alcohols

Secondary heptyl alcohols. Four of these alcohols were obtained and all appear to be highly conjugated in rabbits (approx. 60%, see Table 3).

Heptan-2-ol. The glucuronide in this case was isolated by continuous ether extraction. On treatment of an ethereal solution of the gum (2 g.) with *p*-toluidine (2 g.) in ether the *p-toluidine salt* of *β-heptyl-2-D-glucuronide* was obtained as colourless leaflets from ethanol and had m.p. 130° and $[\alpha]_D - 39·7°$ in water (*c*, 1). (Found: C, 59·6; H, 8·2; N, 3·5. $C_{20}H_{33}O_7N$ requires C, 60·1; H, 8·3; N, 3·5%.) The *triacetyl β-heptyl-2-glucuronide methyl ester* formed fine needles, m.p. 80–90°. (Found: C, 54·9; H, 7·3. $C_{20}H_{32}O_{10}$ requires C, 55·6; H, 7·5%.) On hydrolysis of the triacetyl methyl ester (0·75 g.) by refluxing for 7 hr. with N-HCl (50 ml.), heptan-2-ol was liberated and identified as the 3:5-dinitrobenzoate, m.p. and mixed m.p. 49° (authentic heptan-2-ol 3:5-dinitrobenzoate has m.p. 49·4°; see Huntress & Mulliken (1946)).

The expired air of a rabbit which had received 5 ml. of heptan-2-ol was drawn through Brady's reagent. After 1·5 hr. from dosing the reagent became cloudy, and ketone continued to be exhaled up to 5 hr. The precipitate of heptan-2-one 2:4-dinitrophenylhydrazone (0·4 g.) was recrystallized from 70% ethanol and had m.p. 69° not depressed by authentic material. Allen (1930) gives the m.p. of heptan-2-one (*n*-amyl methyl ketone) 2:4-dinitrophenylhydrazone as 89°. Highly purified synthetic heptan-2-one 2:4-dinitrophenylhydrazone was prepared independently for us by Dr G. King who reports the m.p. as 74·5°. (Found: C, 53·3; H, 6·3; N, 19·2. Calc. for $C_{13}H_{18}O_4N_2$: C, 53·1; H, 6·2; N, 19·0%.) This highly purified material did not depress the m.p. of our urinary material and it appears that the m.p. quoted by Allen (1930) is too high.

Heptan-2-one. The output of glucuronide in three rabbits each receiving 0·95 g./kg. orally of *n*-amyl methyl ketone was found to be 39·9, 41·2 and 42·5% (average 41%) of the dose, respectively. Thus a considerable amount of the ketone was reduced to the alcohol, which was then excreted conjugated. The glucuronide was isolated from these urines as a gum by ether extraction and proved to be heptyl-2-glucuronide by the preparation from it of the *p*-toluidine salt of heptyl-2-glucuronide, m.p. and mixed m.p. 130° and $[\alpha]_D^{20°} - 41·1°$ in water (*c*, 1). (Found: C, 60·1; H, 8·6; N, 3·3. Calc. for $C_{21}H_{33}O_7N$: C, 60·1; H, 8·3; N, 3·5%.) Also the triacetyl β-heptyl-2-glucuronide methyl ester (m.p. and mixed m.p. 80–90° and $[\alpha]_D - 30·6°$ in $CHCl_3$ (*c*, 1)) was made. (Found: C, 55·8; H, 7·5. Calc. for $C_{20}H_{32}O_{10}$: C, 55·6; H, 7·5%.)

The urine also contained small amounts of the unchanged ketone since it gave a weak Rothera test and a faint precipitate with Brady's reagent.

Heptan-3-ol. The glucuronide gum (8 g.) from the urine of three rabbits which had collectively received 9 g. of heptan-3-ol was obtained by continuous extraction with ether for 32 hr. *Triacetyl β-heptyl-3-D-glucuronide methyl ester* formed long fine needles, m.p. 79–80°. (Found: C, 55·5; H, 7·4%.)

On hydrolysis this ester yielded heptan-3-ol, which was identified as the *3:5-dinitrobenzoate*, m.p. and mixed m.p. 51°. (Found: C, 54·8; H, 6·0; N, 9·4. $C_{14}H_{18}O_6N_2$ requires C, 54·2; H, 5·9; N, 9·0%.) The 3:5-dinitrobenzoate of heptan-3-ol appears to be a new compound and it was therefore synthesized from heptan-3-ol, pyridine and 3:5-dinitrobenzoyl chloride. The authentic material formed pale yellow plates from aqueous methanol, m.p. 53°. (Found: C, 54·5; H, 5·9; N, 9·0%.) Its m.p. was depressed by the isomeric 3:5-dinitrobenzoate of heptan-2-ol, m.p. 51°.

Heptan-4-ol (*di-n-propylcarbinol*). The glucuronide gum (4·5 g.) was obtained from the urine of three rabbits which had each received 3 g. of heptan-4-ol, by continuous extraction with ether for 3 hr. This gum was soluble in $CHCl_3$. *Triacetyl β-heptyl-4-D-glucuronide methyl ester* formed very fine colourless needles from aqueous methanol, m.p. 103°. (Found: C, 55·0; H, 7·7%.)

2:4-Dimethylpentan-3-ol (*diisopropylcarbinol*). The urine from three rabbits which had each received 3 g. of the alcohol was extracted for 1 week with ether. The glucuronide gum (7 g.) partly crystallized. It was completely crystallized by dissolution in a little ethanol, adding an excess of benzene and allowing the solution to evaporate slowly. *β-2:4-Dimethylpentyl-3-D-glucuronide* formed colourless needles from ethyl acetate, m.p. 150° and $[\alpha]_D^{25°} - 54° \pm 2°$ in water (*c*, 1). (Found: C, 53·3; H, 8·0. $C_{13}H_{24}O_7$ requires C, 53·4; H, 8·3%.) It was very soluble in water and ethanol, soluble in ether and moderately soluble in ethyl acetate. It formed a *potassium salt* with K_2CO_3 and this, on recrystallization from 95% ethanol, formed colourless needles of the *dihydrate* of $[\alpha]_D^{20°} - 43·3° \pm 1·5°$ in water (*c*, 1). (Found: C, 43·0; H, 7·5; K, 11·3. $C_{13}H_{23}O_7K.2H_2O$ requires C, 42·6; H, 7·4; K, 10·7%.) The *benzylamine salt* of the glucuronide was formed on treating the glucuronide gum (0·3 g.) with benzylamine (0·1 g.). The mixture set solid and was recrystallized from 95% ethanol. This salt (0·23 g.) formed needles, m.p. 195° and $[\alpha]_D - 39·4° \pm 0·5°$ in water (*c*, 1) and was very soluble. (Found: N, 3·3. $C_{20}H_{33}O_7N$ requires N, 3·5%.) *Triacetyl β-2:4-dimethylpentyl-3-D-glucuronide methyl ester* formed colourless needles, m.p. 154°. (Found: C, 55·6; H, 7·5%.)

sec.-*Octyl alcohol* (*octan-2-ol*). This alcohol was a diuretic and yielded a urine which did not reduce Benedict solution but which gave a Rothera test. The ketone (presumably octan-2-one) only occurred in small amounts in the urine and was not isolated. The conjugation of this alcohol with glucuronic acid was low (see Table 3). The glucuronide was isolated as a gum, but neither this nor its triacetyl methyl ester could be induced to crystallize.

Tertiary alcohols

The three tertiary alcohols studied show high conjugation with glucuronic acid, and the glucuronides were readily isolated from the urine.

tert.-*Butyl alcohol.* Six rabbits were each given 8 ml. of the alcohol, and the glucuronide gum (7·2 g.) was isolated from the 24 hr. urine by the basic lead acetate method. On methylation and acetylation the gum yielded *triacetyl β-1:1-dimethylethyl-D-glucuronide methyl ester* (3·76 g. after recrystallization from ethanol/*n*-hexane mixtures), m.p. 168°. (Found: C, 52·3; H, 6·8. $C_{17}H_{26}O_{10}$ requires C, 52·3; H, 6·7%.) No aldehyde or ketone was detected in the expired air of a rabbit which had received 6 ml. of this alcohol.

tert.-*Amyl alcohol.* The glucuronide gum was isolated from the acidified $(NH_4)_2SO_4$-saturated urine by repeated extraction with equal volumes of ethanol/ether (1:4), after feeding 8·25 g. of the alcohol to three rabbits (3 ml. each). On methylation and acetylation of half of the glucuronide gum there was obtained *triacetyl β-1:1-dimethylpropyl-D-glucuronide methyl ester* (2·3 g. pure), m.p. 111°, after recrystallization from ethanol/water. (Found: C, 53·9; H, 7·1. $C_{18}H_{28}O_{10}$ requires C, 53·5; H, 7·0%.)

Estimations of the ethereal sulphate output were also carried out with this alcohol, but no change from normal was noted.

1:1-Dimethylbutan-1-ol (tert.-*hexyl alcohol*). The glucuronide of this alcohol was isolated as the basic lead salt formed with basic lead acetate at pH 9. It was obtained as a water-soluble gum from which was prepared *triacetyl β-1:1-dimethylbutyl-D-glucuronide methyl ester*, m.p. 104°. (Found: C, 54·8; H, 7·2. $C_{19}H_{30}O_{10}$ requires C, 54·5; H, 7·2%.)

DISCUSSION

It appears that aliphatic alcohols are metabolized and eliminated from the body along three paths, namely (1) by oxidation and elimination of the products (acids, aldehydes, ketones and carbon dioxide) in the urine and expired air, (2) by conjugation with glucuronic acid and elimination of the glucuronides in the urine and (3) by elimination of the unchanged alcohol in the expired air or urine. Which of these routes constitutes a major pathway for any particular alcohol will depend on a number of factors. Physical factors such as volatility and dose of alcohol are involved to some extent, but the present work suggests that much depends on chemical factors, such as the number of carbon atoms in the alcohol, the nature of the alcoholic hydroxyl group and the extent of branching of the hydrocarbon chain.

It seems that, on the whole, the lower alcohols, e.g. methanol (Lund, 1948), ethanol (Bartlett & Barnet, 1949), amyl alcohol (Haggard *et al.* 1945) are extensively metabolized before being eliminated, since only small amounts are eliminated unchanged at doses of about 1 g./kg. The two processes involved are oxidation and conjugation, and the results reported here suggest that the portion of the alcohol not oxidized is conjugated to form water-soluble glucuronides. Examination of Tables 1–5 shows that the general order of conjugation of primary, secondary and tertiary alcohols is in the order tertiary > secondary > primary, and, since Haggard *et al.* (1945) have shown that oxidation is in the reverse order to this, it follows that the portion of alcohol not oxidized is probably conjugated.

Normal primary alcohols. These alcohols are in general readily oxidized in the body, via aldehydes, to the corresponding acids and to carbon dioxide (e.g. Stotz, 1943, on ethanol; Orskov, 1950, on propanol; Haggard *et al.* 1945, on amyl alcohols). The lower alcohols, methanol and ethanol, only give appreciable amounts of glucuronides when given in large doses (Kamil, Smith & Williams, 1952), but as the chain length increases the conjugation is more apparent, although still less than 10%. This conjugation has been proved by isolation of the glucuronides of methanol, ethanol and *n*-hexanol.

Arborescent primary alcohols. These are discussed in the succeeding paper (Kamil *et al.* 1953).

Secondary alcohols. The main metabolic route of the secondary alcohols may be written

$$RR'CHOH \begin{cases} \rightarrow RR'CO \\ \rightarrow RR'CHOC_6H_9O_6 \end{cases}.$$

In the three cases examined, *iso*propanol, *sec.*-butanol and heptan-2-ol, ketones were found in the expired air, and all the alcohols are conjugated to some extent with glucuronic acid, the amount increasing with molecular weight up to 2-heptanol. Since our observations did not extend beyond 2-octanol we cannot say if this is an exception or whether we have a peak of conjugation at seven carbon atoms.

The reaction $RR'CHOH \rightarrow RR'CO$ appears readily reversible *in vivo*. Heptan-2-one gave rise to heptyl-2-glucuronide (42% of the dose by quantitative estimations), and this was found to be identical with the glucuronide obtained on feeding DL-heptan-2-ol. No evidence was found of any asymmetrical reduction of the ketone, but the optical activity of heptan-2-ol is so small ($[\alpha]_D$ about $\pm 11°$) that it would have been difficult to detect any resolutions in our preparations.

Tertiary alcohols. Oxidation here could only occur by oxidizing the hydrocarbon groups or disrupting the molecule and with this increased difficulty of oxidation the conjugations (Table 4) are high.

Optical rotations of the triacetyl methyl esters

The rotations of these derivatives in chloroform are quoted in Table 6 and have values ranging from $-30°$ to $-40·9°$, which are not very different from the synthetic triacetyl-β-methyl-D-glucuronide methyl ester (Goebel & Babers, 1935). No attempt was made to resolve glucuronides produced from those alcohols having an asymmetric carbon atom, which could have been mixtures of different proportions of two diastereoisomers. Since, however, the optical forms of the alcohols have low rotations, not more than $\pm 10°$, the rotations of these diastereoisomeric glucuronides are not likely to be very different. This can be seen from Table 6. The optically inactive alcohols are *iso*propanol, pentan-3-ol, heptan-4-ol and 2:4-dimethylpentan-3-ol, and the specific rotations of the triacetyl methyl esters are, respectively, $-37·8°$, $-33·3°$, $-37·7°$ and $-40·9°$. All derivatives of the optically active alcohols are not far from this range, the lowest being the glucuronide of heptan-2-ol, $-31·6°$, and the highest that of 2-methylpentan-2-ol, $-47·3°$.

Table 6. *The optical rotations of* 1% *solutions in chloroform of the triacetyl methyl esters of aliphatic* β*-glucuronides*

β-Glucuronide	M.p. (°)	$[\alpha]_D^{20°}$ (*c*, 1)	$[M]_D/100$
Primary alcohols:			
Methyl*	148 (149–150)†	−30·3 (−28·9)†	−105
Ethyl*	144	−32·8	−118
n-Hexyl	70–71	−31·8	−133
*iso*Amyl	96	−30	−121
Secondary alcohols:			
*iso*Propyl	140	−37·8	−132
Butyl-2-	107	−37·0	−144
Pentyl-2-	90	−44·2	−179
Pentyl-3-	76	−33·3	−135
Hexyl-2-	80–85	−37·2	−155
4-Methylpentyl-2-	119	−47·3	−198
Heptyl-2-	78	−31·6	−137
Heptyl-3-	79–80	−35·7	−154
Heptyl-4-	103	−37·7	−163
2:4-Dimethylpentyl-3-	154	−40·9	−177
Tertiary alcohols:			
tert.-Butyl	168	−29·2	−114
tert.-Amyl	111	−36·6	−148
1:1-Dimethylbutyl (*tert.*-hexyl)	104	−27·4	−115

* Kamil *et al.* (1952 and unpublished observations).

† Recorded by Goebel & Babers (1935) for the synthetic compound.

SUMMARY

1. A study has been made of the glucuronic acid conjugation in the rabbit of thirty aliphatic alcohols. All were found to conjugate to some extent.

2. The conjugation of normal primary alcohols from methyl to decyl was found to be low, being less than 10% of the dose, but its occurrence was proved by isolation.

3. The conjugation of secondary alcohols increased with increasing number of carbon atoms in the molecule from 10% with *iso*propanol to 60–70% for the secondary heptyl alcohols. With octan-2-ol the conjugation was low. The oxidation products of some of the secondary alcohols (*iso*propanol, butan-2-ol and heptan-2-ol) were proved to be ketones.

4. The conjugation of three tertiary alcohols, butyl, amyl and hexyl, was higher than that of the corresponding primary and secondary alcohols.

5. The results suggest that conjugation is an alternative metabolic process to oxidation. The order of conjugation is tertiary > secondary > primary, which is the reverse of the order for ease of oxidation.

6. In most cases the glucuronides were isolated and characterized as triacetyl methyl esters.

7. Aliphatic alcohols do not appear to form ethereal sulphates.

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Studies in Detoxication

47. THE FORMATION OF ESTER GLUCURONIDES OF ALIPHATIC ACIDS DURING THE METABOLISM OF 2-ETHYLBUTANOL AND 2-ETHYLHEXANOL

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The octyl alcohol, DL-2-ethyl-*n*-hexanol, is used industrially as a solvent and, in the form of the ester, di-2-ethylhexyl phthalate, as a plasticizer. According to Shaffer, Carpenter & Smyth (1945) this ester is saponified in rats and rabbits, phthalic acid being excreted and there being also an increase in the short-chain fatty acids in the urine. After feeding the DL-alcohol to rabbits, Akiya & Yamakawa (1950) isolated α-ethylhexanoic acid (α-ethylcaproic) from the urine, showing a slight positive rotation. They also found a dibasic acid with a slight positive rotation. No reports of the formation of glucuronide from the alcohol have been made. The toxicity of 2-ethylhexanol is comparable with that of *iso*octyl alcohol, the anaesthetic dose of both alcohols being near the lethal dose in rats (Nelson, 1951).

It has thus been proved that 2-ethylhexanol is oxidized *in vivo* to the corresponding fatty acid and it would be expected that 2-ethylbutanol would be similarly oxidized to diethylacetic acid (α-ethylbutyric acid). Blum & Koppel (1911) injected diethylacetic acid into a dog and reported methyl *n*-propyl ketone in the urine. Dziewiatkowski, Venkataraman & Lewis (1949) also fed and injected this acid into rats and rabbits, but were unable to detect any ketone in the urine. They did, however, observe that both diethylacetic and 2-ethylhexanoic acids gave rise to copper-reducing urines and extra glucuronic acid. This suggested that these acids were being excreted partly as ester glucuronides. In the rabbit extra glucuronic acid corresponding to 25–52 % of the dose of diethylacetic acid and to 83–87 % of the α-ethylhexanoic acid was excreted. The ester glucuronides, however, were not isolated.

In the present work it has been found that 2-ethylbutanol and 2-ethylhexanol give rise in the rabbit to the excretion of the ester glucuronides of diethylacetic and α-ethylhexanoic acids respectively. These glucuronides have been isolated and appear to be the first ester glucuronides of fatty acids to be described.

EXPERIMENTAL

In the preceding paper (Kamil, Smith & Williams, 1953) it was shown that extra glucuronic acid was excreted by rabbits after dosing with 2-ethylbutanol and 2-ethylhexanol. The glucuronic acid excretion corresponded to 40 % of the dose of 2-ethylbutanol and 87 % of 2-ethylhexanol (dose 25 m-moles/3 kg. rabbit).

Metabolism of 2-ethylbutan-1-ol

Isolation of diethylacetic acid and methyl propyl ketone. A rabbit was given 2·55 g. (3·1 ml.) of the alcohol and a 24 hr. urine was collected. This urine was strongly reducing and gave a strong positive naphthoresorcinol test which indicated the presence of a reducing ester glucuronide. The urine was acidified, treated with 100 ml. of Brady's reagent and kept for 24 hr. The precipitate which had formed was filtered and recrystallized from ethyl acetate. It proved to be methyl *n*-propyl ketone 2:4-dinitrophenylhydrazone (yield, 7 mg.), m.p. and mixed m.p. 140°. (Found: N, 20·6. Calc. for $C_{11}H_{14}N_4O_4$: N, 21·0 %.) The filtrate from the hydrazone was made 2N with respect to Na_2CO_3 by adding solid Na_2CO_3, and then boiled for 0·5 hr. After cooling, the solution was acidified with H_2SO_4 and extracted in a funnel with 2 × 200 ml. of ether. The extract was dried over $CaCl_2$, evaporated and the oily residue distilled. The fraction boiling at 140–200° was collected and heated with an equal weight of thionyl chloride till the reaction ceased. The product was then treated with an equal weight of *p*-toluidine, warmed and the product recrystallized from a large volume of hot water. In this way, 0·5 g. of the *p*-toluidide of diethylacetic acid, m.p. and mixed m.p. 117° (m.p. 116·2° is quoted by Huntress & Mulliken, 1946), was isolated. (Found: C, 75·6; H, 9·4; N, 7·0. Calc. for $C_{13}H_{19}NO$: C, 76·0; H, 9·3; N, 6·8 %.)

Isolation of diethylacetylglucuronide. From the 24 hr. urine of four rabbits which had collectively received 14 ml. of diethylethanol, there were obtained through the basic lead salt 13 g. of glucuronide gum which could not be crystallized. The potassium salt of β-diethylacetylglucuronide (0·25 g.) was obtained in poor yield by mixing 1·2 g. of the gum, 70 mg. K_2CO_3 and a few drops of water, then diluting with a few ml. of ethanol and filtering. The filtrate was diluted to 50 ml. with 98 % ethanol and on keeping at 0°, the *potassium salt* separated as

rectangular plates which reduced Fehling's solution on boiling. These decomposed at 168–170° and showed $[\alpha]_D^{20°} - 27{\cdot}1°$ in water (*c*, 0·8). (Found: C, 41·5; H, 6·2; K, 12·0; glucuronic acid, $C_6H_{10}O_7$, 52·3. $C_{12}H_{19}O_8K.H_2O$ requires C, 41·4; H, 6·0; K, 11·2; $C_6H_{10}O_7$, 55·7 %.)

The gum also yielded on methylation and acetylation *triacetyl β-diethylacetyl-*D*-glucuronide methyl ester* in poor yield. This formed long fine needles from *n*-hexane; it was optically inactive in $CHCl_3$ and had m.p. 72°. (Found: C, 52·9; H, 6·5. $C_{19}H_{28}O_{11}$ requires C, 52·7; H, 6·5 %.) The compound was insoluble in water and reduced Benedict's reagent on heating when acetone was added to assist its solution.

The metabolism of DL*-2-ethylhexan-1-ol*

The urine of rabbits receiving 2-ethylhexanol strongly reduced copper reagents and this, together with an intense naphthoresorcinol reaction, suggested the formation of an ester glucuronide which was isolated.

Isolation of α-ethylhexanoylglucuronide. Three rabbits were each given 3 ml. of 2-ethylhexanol by stomach tube. The 24 hr. urine was acidified to pH 4 with HCl and continuously extracted with ether for 8 hr., when the extraction was complete. Evaporation of the ether gave the glucuronide gum (5 g.). This was treated with 1 g. of K_2CO_3 and when effervescence had ceased the residue was dissolved in warm ethanol (50 ml.). On standing, *potassium β-2-ethylhexanoyl-*D*-glucuronide monohydrate* separated (2 g.). It formed white needles from ethanol, m.p. 170° decomp. and $[\alpha]_D - 27{\cdot}5°$ in water (*c*, 0·6). (Found: C, 44·2; H, 6·5; K, 10·6. $C_{14}H_{23}O_8K.H_2O$ requires C, 44·7; H, 6·7; K, 10·4 %.) It reduced Benedict's and Fehling's solution on heating. *Triacetyl β-2-ethylhexanoyl-*D*-glucuronide methyl ester* formed colourless needles from hexane, m.p. 82°. (Found: C, 55·2; H, 7·0. $C_{21}H_{32}O_{11}$ requires C, 54·8; H, 7·0 %.) This ester was optically inactive in chloroform and reduced Benedict's solution on heating with a little acetone to assist solution.

Isolation of α-ethylhexanoic acid. Three rabbits were given 3 ml. each of 2-ethylhexanol and the 24 hr. urine (260 ml.) treated with 8 ml. conc. HCl. The solution was heated to boiling for 15 min., cooled and extracted with ether (250 ml.). The extract, which reduced Benedict's reagent, was evaporated and the residue dissolved in water. α-Ethylhexanoic acid was extracted with light petroleum (b.p. 40–60°) in which the glucuronide is insoluble. The light petroleum was evaporated and the residual oil distilled. The main fraction (1 ml.) distilled at 225° and the undiluted oil showed $[\alpha]_D^{20°} - 1{\cdot}2° \pm 0{\cdot}05$. The α-ethylhexanoic acid was thus optically active. The optical isomers of α-ethylhexanoic acid have been prepared by Kenyon & Platt (1939) who record $[\alpha]_D^{20°} + 8{\cdot}95°$ and $-8{\cdot}75°$. It would thus appear that our material contains roughly 56 % (−) isomer and 44 % (+) isomer. On treatment with thionyl chloride followed by ammonium hydroxide the acid yielded crystalline α-ethyl-*n*-hexanoamide, m.p. 103–104° (from ligroin), mixed m.p. 104°, with authentic α-ethyl-*n*-hexanoamide, m.p. 104–105° (Magnani & McElvain, 1938).

2-Ethylhexanol urine also contained traces of a ketone, which could be di-*n*-propyl ketone or *n*-amyl methyl ketone, but the amounts present were too small for satisfactory identification. An attempt was made to detect this ketone in the breath of rabbits. A rabbit was given 6 ml. of 2-ethylhexanol and placed in the Perspex tank mentioned earlier. The expired air was drawn through Brady's reagent for 24 hr. but no ketone was detected.

DISCUSSION

In the preceding paper (Kamil *et al.* 1953) it was suggested that the glucuronic acid conjugation of primary aliphatic alcohols was low, probably because of their rapid oxidation. The glucuronic acid excretion after feeding 2-ethylbutanol and 2-ethylhexanol is, however, very high and it has been shown that this is due, not to the conjugation of the alcohols themselves with glucuronic acid, but to the conjugation of their oxidation products. The formation and isolation of the ester glucuronides of diethylacetic and α-ethylhexanoic acids after feeding the corresponding alcohols thus shows that the primary aliphatic alcohols are rapidly oxidized *in vivo* and that the low percentage of ether glucuronide formation is due to the rapidity of this oxidation.

In all we have studied four 2-substituted primary alcohols; two of them, *iso*butanol and 2-methylbutanol, carry 2-methyl groups and are of the general type $RCH(CH_3)CH_2OH$, whereas the other two, 2-ethylbutanol and 2-ethylhexanol, carry 2-ethyl groups and are of the general type

$$RCH(C_2H_5)CH_2OH.$$

The 2-methyl-substituted alcohols should give rise to α-methyl fatty acids, $RCH(CH_3)COOH$, which are readily oxidized *in vivo*. On the other hand, the 2-ethyl-substituted alcohols give rise to α-ethyl fatty acids which are not readily oxidized *in vivo*. In fact, α-substituted fatty acids in which the substituent is larger than the methyl group are not readily oxidized in the body (for discussion see Williams (1947) and Breusch (1948)). This difference in the oxidation of the α-methyl and α-ethyl fatty acids is reflected in the glucuronic acid conjugation.

The main route of metabolism of the 2-methyl primary alcohols appears to be

$$RCH(CH_3)CH_2OH \rightarrow RCH(CH_3)COOH \rightarrow$$

Table 1. *Optical rotations of aliphatic ester glucuronides*

β-Glucuronide	Potassium salt $[\alpha]_D$ in water	Triacetyl methyl ester M.p. (°)	Triacetyl methyl ester $[\alpha]_D$ in $CHCl_3$	Triacetyl methyl ester $[M]_D/100$ in $CHCl_3$
Acetyl*	—	172	+8·7	+32
α-Diethylacetyl	−27·1	72	0	0
α-Ethylhexanoyl	−27·5	82	0	0

* Synthetic (Goebel & Babers, 1934).

further oxidation products. There is some formation of the glucuronide, $RCH(CH_3)CH_2O.C_6H_9O_6$. With the 2-ethyl alcohols, the main route of metabolism is

$$RCH(C_2H_5)CH_2OH \rightarrow RCH(C_2H_5)COOH \rightarrow RCH(C_2H_5)CO.O.C_6H_9O_6.$$

The product of oxidation of 2-ethylbutanol, however, is not completely resistant to oxidation, for a small amount of methyl *n*-propyl ketone was isolated from the urine. This ketone was also isolated by Blum & Koppel (1911) from the urine of a dog injected with diethylacetic acid, and presumably arises by decarboxylation of α-ethylacetoacetic acid, the β-oxidation product of diethylacetic acid. The metabolism of diethylethanol in the rabbit is thus mainly as follows:

$$(C_2H_5)_2CHCH_2OH \rightarrow (C_2H_5)_2CHCOOH \rightarrow (C_2H_5)_2CHCOOC_6H_9O_6$$
$$\downarrow$$
$$CH_3COCH(C_2H_5)COOH \rightarrow CH_3COCH_2C_2H_5$$

2-Ethylhexanol gives rise to very large outputs of glucuronide, and nearly 90% of the dose can be accounted for as α-ethylhexanoylglucuronide. Its main route of metabolism in the rabbit is thus

$$C_4H_9CH(C_2H_5)CH_2OH \rightarrow C_4H_9CH(C_2H_5)COOH \rightarrow C_4H_9CH(C_2H_5)COOC_6H_9O_6.$$

According to Akiya & Yamakawa (1950) there is a further oxidation to a dibasic acid, and the present work suggests that a trace of ketone is also excreted. This ketone would be interesting if it could be isolated, since it could be either amyl methyl ketone or di-*n*-propyl ketone, or a mixture of both. Since 2-ethylhexanol contains an asymmetric carbon atom there is a possibility of resolution. The α-ethylhexanoic acid we isolated did in fact show optical activity corresponding to a mixture of 56% (−) isomer and 44% (+) isomer. At the moment, however, it cannot be decided whether the slight resolution had occurred biologically or chemically during isolation. Since 2-ethylhexanol gives very high outputs of ester glucuronide we believe that the resolution must have been the result of chemical manipulations. This point also raises the question of the nature of α-ethylhexanoylglucuronide, since the aglycone could be the (−) or (+) isomer or an equal or unequal mixture of these. The optical rotations of the known ester β-glucuronides of aliphatic acids are given in Table 1, the acetyl derivative being synthetic (Goebel & Babers, 1934). The potassium salts of both diethylacetyl- and α-ethylhexanoylglucuronides have nearly the same rotation and this is also true of the triacetyl methyl esters. Since diethylacetic acid is optically inactive, the rotation of its glucuronide is that of an aliphatic ester glucuronide carrying an optically inactive aglycone. Since the glucuronide of α-ethylhexanoylglucuronide has approximately the same rotation, it is reasonable to conclude that here again the aglycone is inactive and that the glucuronide can be described as (±)-α-ethylhexanoyl-β-D-glucuronide, which is a partially racemic compound of the Ladenburg type. This is further supported by the fact that the triacetyl methyl ester has a sharp melting point.

Diethylacetyl- and α-ethylhexanoyl-glucuronides are the first ester glucuronides of fatty acids to be described. Ester glucuronides of the aromatic acids (e.g. benzoylglucuronide) are well known. It is noteworthy that these two aliphatic ester glucuronides appear to be much more stable than those of the aromatic series and only reduce Benedict's reagent relatively slowly.

SUMMARY

1. 2-Ethylbutanol is excreted by rabbits as an ester glucuronide which was proved by isolation to be diethylacetylglucuronide. A small amount of methyl *n*-propyl ketone is also excreted and this was isolated as the 2:4-dinitrophenylhydrazone.

2. Nearly 90% of DL-2-ethylhexanol is excreted by rabbits as α-ethylhexanoylglucuronide which was isolated and characterized as the potassium salt and triacetyl methyl ester.

3. The results are discussed and it is suggested that ester glucuronides may be formed from branched-chain fatty acids when β-oxidation is blocked.

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Studies on the Metabolism of Semen

8. ERGOTHIONEINE AS A NORMAL CONSTITUENT OF BOAR SEMINAL PLASMA. PURIFICATION AND CRYSTALLIZATION. SITE OF FORMATION AND FUNCTION

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The discovery of ergothioneine is due to Tanret (1909) who isolated it from ergot of rye and established its composition as $C_9H_{15}N_3O_2S$. Barger & Ewins (1911) identified the new substance as a betaine of thiolhistidine (β-2-thiolglyoxaline-4(5)-propiobetaine). They were also the first to point out that the sulphur of thiolglyoxaline is readily oxidized by ferric chloride or bromine to free sulphuric acid; this property, which distinguishes ergothioneine from cysteine and glutathione, has been utilized for the determination of ergothioneine (Blumenthal & Clarke, 1935; Touster, 1951). The final confirmation of the structure of ergothioneine was provided by Heath, Lawson & Rimington (1950, 1951) who succeeded in synthesizing ergothioneine from 2-thiolhistidine, an amino-acid which had been obtained synthetically (Ashley & Harington, 1930; Harington & Overhoff, 1933) but has hitherto never been found in nature and which is unable to replace histidine as a growth-promoting factor in animals (Neuberger & Webster, 1946).

Ergot, from which ergothioneine can be obtained in yields varying from 65 to 260 mg./100 g. (Eagles, 1928; Pirie, 1933*a*; Hunter, Molnar & Wight, 1949), remained the only known source of ergothioneine until Hunter & Eagles (1925, 1927) isolated from pig blood the crystalline substance X, later renamed sympectothion which, although different from uric acid, gave with phosphotungstic and arsenophosphotungstic acid reagents the same blue colour reaction as uric acid. A blood constituent with similar properties, named thiasine, was isolated independently by Benedict, Newton & Behre (1926). Both sympectothion and thiasine were soon shown to be identical with ergothioneine (Newton, Benedict & Dakin, 1926; Eagles & Johnson, 1927). Blood ergothioneine, or 'thioneine' as it is sometimes called, is confined entirely to the erythrocytes and does not occur in the plasma. A sensitive and specific method for its estimation based on the diazo reaction has been developed by Hunter (1928, 1949). The content of ergothioneine in blood (mg./100 ml.) is much smaller than in ergot; it varies from 1·8 to 1·95 in man, 1·3 to 3·1 in rat, and 2·0 to 26·5 in pig (Hunter, 1951).

The present study reports the discovery of a third source of ergothioneine in nature, namely the seminal plasma (preliminary communication: Leone & Mann, 1951). Protein-free extracts from semen have been known for a long time to exhibit a marked reducing property towards 2:6-dichlorophenolindophenol, but this fact has usually been attributed to ascorbic acid (cf. Berg, Huggins & Hodges, 1941). We were able to confirm the occurrence of ascorbic acid in semen, but in addition we detected the presence of yet another reducing substance which has hitherto escaped recognition by other workers. This substance is ergothioneine. It occurs in a particularly high concentration in the semen of boar, and it originates in the seminal vesicles, the glands which also secrete fructose (Mann, 1946), citric acid (Humphrey & Mann, .1949) and inositol (Mann, 1951). In the secretion of the seminal vesicles, ergothioneine is present in a dialysable form. The

* British Council Scholar.

Concentration of ergothioneine in the boar vesicular secretion is much higher than in the blood of any species including pig, and in some instances was found to approach the level in ergot. Thus, boar vesicular secretion represents a convenient starting material for the preparation of crystalline ergothioneine. It is important to stress that, unlike blood, the semen carries ergothioneine as an extracellular constituent.

MATERIAL AND METHODS

Boar semen and vesicular secretion. A single boar semen ejaculate varies from 150 to 500 ml., but has only a low density of spermatozoa (25000–300000/μl.) and its large bulk is made up by the seminal plasma. According to McKenzie, Miller & Bauguess (1938), the relative contributions to the semen volume are: 15–25% by the seminal vesicles, 10–20% by Cowper's glands, 55–70% by the prostate and urethral glands and only 2–5% by sperm and epididymal fluid. In the present study, whole semen, as ejaculated, was collected by means of an artificial vagina. Epididymal semen, i.e. the thick sperm suspension stored in the cauda epididymidis, was obtained from the glands by incision; the sperm density of the epididymal semen was 2000000–5000000/μl. The vesicular secretion was removed, either by incision or by means of a syringe, from the vesicles of freshly slaughtered animals; the seminal vesicles, the weight of which varied from 100 to 920 g., contained between 70 and 560 ml. secretion.

Ram and bull semen were collected by means of an artificial vagina (Walton, 1945). The semen was used whole or it was separated by centrifugation into seminal plasma and spermatozoa. The preparation of washed sperm suspensions was carried out as previously described (Mann, 1946).

Quantitative determination of ergothioneine. The method used as a routine for the quantitative determination of ergothioneine in whole semen, seminal plasma and in the male accessory gland secretions, was that described by Hunter (1949), and the diazo reaction was carried out following closely Hunter's (1951) procedure in extracts prepared by deproteinization with either $Zn(OH)_2$ or 66–80% ethanol. Occasionally parallel estimations were made, some by the method of Brown (1945) which depends on the Folin-Marenzi reaction of ergothioneine with the 'uric acid reagent', and others by the method of Touster (1951) in which the thiol group is oxidized with Br_2 to sulphate, which is next precipitated as benzidine sulphate and the benzidine content of the precipitate determined colorimetrically with sodium 1:2-naphthaquinone-4-sulphonate, according to Letonoff & Reinhold (1936).

Chromatographic detection of ergothioneine, and differentiation from thiolhistidine, uric acid and ascorbic acid

Paper partition chromatography has proved a useful means for the detection of small amounts of ergothioneine (5μg. or even less). Chromatograms were run at room temperature for 24 hr. on Whatman no. 1 filter paper, with butanol-acetic acid as solvent (40 vol. *n*-butanol + 10 vol. acetic acid + 50 vol. water; upper layer used). The spots were located on the dry paper chromatograms by photographic detection of ultraviolet-absorbing areas, following a procedure which is similar to that described by Markham & Smith (1949) for the chromatographic detection of purines; the source of ultraviolet light was a mercury arc lamp with a high emission at 254 and 265 mμ., and the photographic paper used was Kodak reflex document paper. When the photographic images were obtained on the contact print, the areas on the paper chromatogram corresponding to the spots on the photographic print were marked. The paper chromatogram thus obtained was sprayed with one of the reagents which give colour reactions with ergothioneine, thiolhistidine, uric acid or ascorbic acid. The following reagents were used.

Diazo reagent (Pauly, 1904; Koessler & Hanke, 1919). The reagent was prepared by mixing immediately before use 2 parts of a solution containing 9 g. sulphanilic acid and 90 ml. conc. HCl/l. with 1 part of 5% (w/v) $NaNO_2$ and 1 part of 20% (w/v) NaOH. The spot produced by diazotized sulphanilic acid with ergothioneine is bright red, with thiolhistidine light orange and with histidine bright yellow with a red margin.

Phosphotungstic acid reagent (Folin & Marenzi, 1929; Work, 1949). The paper chromatogram was sprayed first with a solution prepared by mixing equal volumes of 50% (w/v) urea and 12% (w/v) NaCN, and a few minutes later with a phosphotungstic acid solution prepared according to Brown (1945). Both ergothioneine and thiolhistidine produced a blue coloration with the phosphotungstic acid reagent in a manner similar to uric acid from which, however, they could be distinguished by the position of the spots.

2:6-*Dichloroquinone-chloroimide* (Fearon, 1944; McAllister, 1951). Ergothioneine was identified and distinguished from uric acid by spraying the paper chromatogram first with 0·125N-Na_2CO_3 and then with a 0·4% (w/v) ethanolic solution of 2:6-dichloroquinone-chloroimide. The colour produced was crimson red with ergothioneine; uric acid gave a yellow-orange spot.

Ammoniacal silver nitrate spray (Trevelyan, Procter & Harrison, 1950). The reagent as used for the detection of sugars reacted also with ergothioneine, to give a characteristic pale spot in contrast to the much darker spots due to various sugars and inositol.

2:6-*Dichlorophenolindophenol* (Mapson & Partridge, 1949). This reagent when applied to paper chromatograms makes it possible to distinguish ergothioneine from other reducing substances, in particular, ascorbic acid. In a similar category was the ferric sulphate reagent used by Strohecker & Sierp (1950) for ascorbic acid determination; this reagent was applied by spraying the paper first with 10% (w/v) KCNS and a few minutes later with a solution which was prepared by dissolving 0·38 g. ferric sulphate in water, adding 2 ml. 20% (v/v) H_2SO_4, and diluting with water to 400 ml.

Ninhydrin, known to react with thiolhistidine (Dent, 1948), gave a completely negative reaction with ergothioneine.

Other analytical methods

Fructose and fructolysis in semen were assayed as described before (Mann, 1948). Lactic acid was determined by the method of Friedemann, Cotonio & Shaffer (1929). The O_2 uptake of spermatozoa was determined in Barcroft manometers. Thiocyanate was determined according to Goldstein (1950), ascorbic acid by the method of Roe & Kuether (1943).

RESULTS

Purification and crystallization of ergothioneine from the boar vesicular secretion

The vesicular secretion as collected directly from the boar seminal vesicle is a milky, highly viscous fluid, the specific gravity of which is usually between 1·04 and 1·06. The dry weight content is of the order of 15–20%, of which as much as one-fourth or even one-third is made up of dialysable material. Out of the several methods tested, the two outlined below were finally chosen for the isolation of ergothioneine from the vesicular secretion as yielding the most satisfactory results.

Method 1. The vesicular secretion is diluted with 2 vol. of water, and the bulky mucous precipitate removed 15 min. later by centrifugation. The centrifuged supernatant is deproteinized with $Zn(OH)_2$ by treating it with 1/10 vol. of 5% (w/v) $ZnSO_4.7H_2O$ and neutralizing with N-NaOH dropwise, with constant stirring. The deproteinized mixture is then immersed in boiling water and the stirring continued long enough to maintain the temperature at 93–97° for at least 2 min., before filtering through coarse filter paper. The filtrate, which should be water-clear or only very slightly opalescent, is made 0·5N by the addition of the required quantity of 10N-H_2SO_4, the precipitate (if any) removed by centrifugation, the supernatant solution cooled to about 10° and treated with 50% solution of phosphotungstic acid (w/v) in 0·5N-H_2SO_4 until the precipitation is complete. After standing overnight in the refrigerator, the precipitate, which contains ergothioneine, is separated by centrifugation, washed with cold 0·5% solution of phosphotungstic acid in 0·5N-H_2SO_4, resuspended in a small amount of water, and ground in a mortar with powdered $Ba(OH)_2$ until the mixture becomes permanently alkaline (blue to thymol blue). It is then centrifuged, the excess of Ba^{2+} removed by addition of H_2SO_4 until the reaction becomes slightly acid (orange to methyl red), spun again, and to the supernatant solution 4 vol. of ethanol added. After standing overnight at room temperature, the ethanol-precipitable material is removed by centrifugation, and the clear, slightly yellowish solution is concentrated to a syrup in a crystallizing dish over phosphorus pentoxide. On gradual addition of ethanol and cooling, crystallization sets in. The first crystalline fraction is obtained by allowing the crystallization to proceed in the presence of 50% ethanol for 24 hr. at 0°. After this fraction has been collected, the ethanol concentration in the filtrate is increased to 75% when a second crop of crystals comes down. The final, third fraction is obtained in presence of 90% ethanol. Each fraction is washed with ethanol and dried, and analysed separately for ergothioneine content. The second crystalline fraction showed the highest absolute content and concentration of ergothioneine.

With the use of the above described procedure, and starting in each case from 1300 ml. of the boar vesicular secretion, we have carried out the isolation of ergothioneine on two separate occasions, and obtained each time about 0·7 g. crystalline material 80% pure.

Method 2. The boar vesicular secretion is treated without any preliminary extraction, with 3 vol. of ethanol, centrifuged, and filtered if necessary. The ethanolic extract is concentrated *in vacuo* at 45° to one-tenth of the original volume, centrifuged, and precipitated with 4 vol. of ethanol. Hardly any ergothioneine is precipitated under these conditions. The ethanolic extract is concentrated *in vacuo* until ethanol is removed and in the aqueous solution ergothioneine is precipitated with phosphotungstic acid in the presence of 0·5N-H_2SO_4. From here onwards, the steps of purification are essentially the same as in method 1; the yield and purity of ergothioneine fractions obtained in this manner are similar to those stated before.

Final purification and identification of ergothioneine. The final purification of seminal ergothioneine depended entirely on repeated recrystallization from 66–70% ethanol. 0·48 g. pure ergothioneine was obtained from 0·7 g. of the 80% pure preparation. The composition of the purest preparation was C, 47·1; H, 6·6; N, 18·2; S, 14·0. Calc. for $C_9H_{15}N_3O_2S$: C, 47·1; H, 6·6; N, 18·3; S, 14·0%. The analysis of total sulphur (carried out by Drs Weiler and Strauss) and the analysis of bromine-oxidizable sulphur by the method of Touster (1951) gave identical results; inorganic sulphate was altogether absent. The nitroprusside test and the ninhydrin test were negative. The phosphorus content was nil. Chromatography revealed no trace of thiolhistidine or of any other ultraviolet-absorbing material. On treatment with potassium hydroxide, carried out according to Barger & Ewins (1911), trimethylamine was obtained. Treatment with mercuric chloride, following the method of Tanret (1909), gave the crystalline Hg derivative of ergothioneine. All principal reactions of crystalline seminal ergothioneine including the diazo, phosphotungstic and dichlorophenolindophenol reactions were compared with similar reactions given by pure synthetic ergothioneine and pure ergothioneine obtained from ergot. No difference was found in their behaviour.

Content of ergothioneine in reproductive organs, semen and other tissues and body fluids

Content of ergothioneine in the boar vesicular secretion. The quantitative determination of ergothioneine in vesicular secretion was carried out on samples from twenty boars. The contents ranged from 29 to 256 mg./100 ml., but out of twenty values, fifteen were within the range of 50–100, and the average figure calculated from all results was 79 mg./100 ml. In most cases, the value obtained by Hunter's diazo method was only a little less than the figure calculated on the basis of Brown's phosphotungstic acid reduction method. Moreover, ergothioneine, as determined by the diazo method, accounted for the major portion of the entire reducing power as assessed by titration with dichlorophenolindophenol. The difference between the total reduction value and that due to ergothioneine was due mostly to ascorbic acid. This can

be seen from the following example in which the total reducing value is expressed as 100 %, and the other results given as percentage of that value.

Total reduction (determined by titration with dichlorophenolindophenol in a trichloroacetic acid-metaphosphoric acid extract from the boar vesicular secretion) ≡ 100 %. Reduction due to ergothioneine determination by the diazo method in the zinc filtrate) ≡ 85 %. Reduction due to ascorbic acid (calculated from the estimation of ascorbic acid by the method of Roe & Kuether, 1943) ≡ 12 %. Reduction due to phosphotungstic acid-reducing substances including ergothioneine (calculated from results obtained by the method of Brown (1945)) ≡ 88 %.

Absence of thiolhistidine in the boar vesicular secretion. In view of the high concentration of ergothioneine in the boar vesicular secretion, and the close chemical relationship between ergothioneine and thiolhistidine, a search was made for thiolhistidine in the vesicular secretion as well as in seminal vesicle tissue itself. To solve the problem, paper chromatography was applied in conjunction with the photographic detection of ultraviolet-absorbing spots. Both thiolhistidine and ergothioneine absorb ultraviolet light, but since their R_F values differ it is possible to resolve mixtures of them completely. However, no thiolhistidine was detected in either the protein-free extracts from the vesicular secretion or in the protein residue, directly or after acid hydrolysis. Similarly, no thiolhistidine was found in acid hydrolysates from the glandular tissue of the seminal vesicles.

Content of ergothioneine in other accessory gland secretions and in boar semen. In contrast to the vesicular secretion, other accessory gland secretions of the boar were found to contain a negligible amount of ergothioneine; boar epididymal semen (representing sperm and the epididymal secretion) 0·2–2 mg./100 ml.; prostatic secretion and Cowper's gland secretion 0·2–1·5 mg./100 ml. Whole boar semen as ejaculated contained on the average about 20 mg. ergothioneine/100 ml. but there were considerable variations between ejaculates even in the same individual. Sperm separated from the boar semen by centrifugation carried only a small content of ergothioneine; the bulk was confined to the seminal plasma.

Urinary ergothioneine. The occurrence of ergothioneine in human urine has been a matter of controversy for some time (Sullivan & Hess, 1933; Work, 1949; Woolf, 1949; Lawson, Morley & Woolf, 1950). An analysis was carried out with concentrates of both human and boar urine prepared according to Work (1949), by means of the chromatographic and detection procedures previously described. The presence of ergothioneine in urine should result in a definite ultraviolet-absorbing area on the paper chromatogram, capable of reacting with the diazo reagent, the phosphotungstic acid reagent and other colour-yielding reagents. Using this procedure, we were able to locate on the chromatograms from urine several spots which were either ultraviolet-absorbing or which reacted with one or the other of the spraying reagents; however, the behaviour of the ultraviolet-absorbing areas in relation to the colour-yielding spots was such as to indicate the occurrence of no more than traces of ergothioneine. This was particularly so in the case of human urine which contained less than 1 mg. ergothioneine/100 ml. On one occasion, boar urine showed some ergothioneine, certainly no more than 2 mg./100 ml., but at all other times less than 1 mg./100 ml. was found.

Blood ergothioneine. The ergothioneine content of boar blood was determined in three animals. The results were 6·1, 3·1 and 4·2 mg./100 ml., respectively.

Ergothioneine in foetal fluids and certain other tissues. The foetal fluids share with the seminal plasma the unusual property of containing fructose as a normal major constituent. However, this chemical resemblance did not extend to ergothioneine. Pig foetal fluids were analysed on two occasions, in a 30-day-old, and in an 80-day-old foetus. There was no ergothioneine in either of these samples. A negative result was also obtained from the analysis of ergothioneine in the pig corpora lutea and adrenal glands, two organs notorious for their high level of ascorbic acid. No ergothioneine could be detected in the pig thyroid gland or in the vitreous fluid of the eye.

The semen of bull, ram and man. Chromatographic analysis of concentrates from bull semen and bull seminal vesicle secretion revealed only a very weak ultraviolet-absorbing and diazo-staining area which could be ascribed to ergothioneine, although at the same time it showed considerable reducing power as assessed by the dichlorophenolindophenol titration and by colour reaction with the phosphotungstic acid 'uric acid reagent'. Ascorbic acid, as estimated by the Roe & Kuether (1943) procedure, accounted for 90 % of the dichlorophenolindophenol reducing substances, but did not account for more than 25 % of the value obtained by the determination with phosphotungstic acid. For the time being, the nature of the phosphotungstic acid-reactive material in bull semen remains obscure. However, a recent investigation by Leone (1952) showed that on treatment of bull-semen concentrates with purified uricase, the colour reaction with phosphotungstic acid markedly decreased. This indicates that some, at least, of the phosphotungstic acid-reactive material is uric acid. Ergothioneine could not be found, except perhaps in traces, in the specimens of

ram and human semen so far examined. On the other hand, in both these species chromatographic analysis revealed the presence in semen of some other diazo-reactive compounds. The relationship between these compounds and ergothioneine remains to be investigated.

Ergothioneine in relation to metabolism and motility of spermatozoa

There is good reason to believe that sulphydryl groups are involved in sperm activity (MacLeod, 1941, 1946; Barron, Nelson & Ardao, 1949), and in the case of human spermatozoa, cysteine and

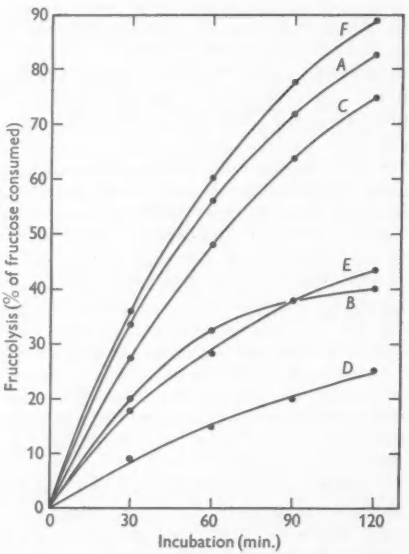

Fig. 1. Anaerobic fructolysis at 37° in ram semen diluted 12 times with Ringer phosphate to which fructose has been added (0·7 mg./ml.). *A*, no additions; *B*, $CuSO_4$ (2×10^{-4} M); *C*, $CuSO_4$ (2×10^{-4} M) + ergothioneine (3×10^{-3} M), added immediately after $CuSO_4$; *D*, H_2O_2 (10^{-3} M); *E*, H_2O_2 (10^{-3} M) + ergothioneine (3×10^{-3} M), added immediately after H_2O_2; *F*, ergothioneine (3×10^{-3} M).

glutathione have both been shown to protect *in vitro* the sperm motility and glycolysis from the inhibitory action of cupric ions (MacLeod, 1951). So far as the boar is concerned, cysteine and glutathione have not been found in the vesicular secretion in any significant quantities. In view, however, of the high content of ergothioneine in the boar vesicular secretion and seminal plasma, it was thought possible that this substance may be capable of protecting spermatozoa against the paralysing action of SH-binding inhibitors.

Experiments were first carried out with ram spermatozoa. The anaerobic fructolysis in dilute ram semen (Fig. 1) was strongly inhibited by 2×10^{-4} M-copper sulphate and by 10^{-3} M-hydrogen peroxide. However, by the simultaneous addition of 3×10^{-3} M-ergothioneine, it was possible to prevent the inhibition, particularly with Cu^{2+}. It will also be noticed that ergothioneine by itself, that is in absence of inhibitors, had a distinctly beneficial influence on the anaerobic rate of fructose utilization. This type of experiment was repeated with the inclusion of a series of semen samples incubated aerobically. Table 1 shows that aerobic fructolysis

Table 1. *Aerobic and anaerobic fructolysis in ram semen*

(Semen diluted 12 times with Ringer phosphate containing 0·7 mg. fructose/ml., and incubated at 37° for 120 min. in air and in N_2 respectively. Fructose content was determined at the end of the incubation period.)

	Decrease in fructose (%)	
Sperm with	Aerobic	Anaerobic
No additions	45	73
Ergothioneine (3×10^{-3} M)	46	76
$CuSO_4$ (2×10^{-4} M)	28	34
$CuSO_4$ + ergothioneine	34	71
H_2O_2 (10^{-3} M)	0	0
H_2O_2 + ergothioneine	5	63

responded to the action of the inhibitors and ergothioneine respectively, in a manner analogous to that demonstrated for the anaerobic process. In this particular experiment, ergothioneine was very effective in counteracting the inhibition caused by hydrogen peroxide. As a rule, however, satisfactory and reproducible results were obtained not so much with hydrogen peroxide, as with Cu^{2+} or with other sulphydryl reagents such as, for instance, *o*-iodosobenzoic acid; 10^{-3} M-sodium iodosobenzoate completely inhibited fructolysis in ram spermatozoa, but the addition to the reaction mixture of 2×10^{-3} M-ergothioneine just before or immediately following the inhibitor, prevented the inhibition. When the addition of ergothioneine was postponed until after the addition of iodosobenzoate, fructolysis could not be fully restored.

Having established the mutually antagonistic action between ergothioneine and the SH group-affecting inhibitors in ram sperm, we carried out a series of experiments with boar sperm. Boar spermatozoa separated from whole ejaculated semen by centrifugation, washed to remove the seminal plasma, and suspended in Ringer phosphate-fructose, utilized fructose at a higher rate in the presence than in the absence of added ergothioneine. Like the fructolysis of ram sperm, that of the boar sperm was sensitive to SH-inhibitors in the absence, but not in the presence, of suitable con-

centrations of ergothioneine. As already mentioned, there is no ergothioneine in the boar epididymis; thus, under physiological conditions, the spermatozoa come in contact with ergothioneine only after having left the epididymis, that is in the course of ejaculation, when they mix with the vesicular secretion. This circumstance offers an excellent opportunity for a comparative study of ejaculated versus epididymal sperm, under the influence of SH-binding inhibitors and ergothioneine,

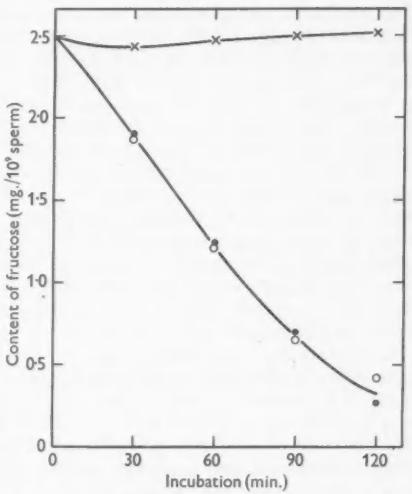

Fig. 2. Anaerobic fructolysis at 37° in boar epididymal spermatozoa to which fructose was added, 2·5 mg. fructose/10^9 sperm; ○—○, no additions; ×— ×, iodosobenzoate (10^{-3} M); ●—●, iodosobenzoate (10^{-3} M) + ergothioneine (2×10^{-3} M).

respectively. Fig. 2 illustrates the result of an experiment carried out with boar sperm taken directly from the epididymis and diluted with Ringer phosphate-fructose. The utilization of fructose by the epididymal spermatozoa was brought to a standstill by 10^{-3} M-iodosobenzoate, but the inhibition could be entirely prevented by the addition of 2×10^{-3} M-ergothioneine, corresponding to 46 mg./100 ml., a concentration of the same order of magnitude as found *in vivo* in the boar vesicular secretion.

Fructolysis measurements in spermatozoa were always carried out in conjunction with observations on motility. It was found that Cu^{2+} and iodosobenzoate exerted the same paralysing effect upon motility as upon anaerobic and aerobic fructolysis; spermatozoa could be protected from loss of motility by the same concentration of ergothioneine as that required for the preservation of fructolysis. This relationship, however, was rather more complicated in the case of another SH-binding inhibitor, namely *p*-chloromercuribenzoate which appeared to affect motility before its influence on the progress of fructolysis became evident.

Some experiments were carried out on similar lines with ergothioneine as an antagonist of SH-inhibitors, in tissues other than semen. It was found, for instance, that lactic acid formation in a Meyerhof extract from rabbit skeletal muscles, which was strongly inhibited by SH-inhibitors, proceeded unchanged in the presence of added ergothioneine. Similarly, SH-inhibitors were unable to exert their inhibitory effect on the alcoholic fermentation in baker's yeast when ergothioneine was present.

With reference to the experiments on the protective action of ergothioneine, it is of interest that the intensity of colour reactions characteristic of ergothioneine such as the diazo reaction, was markedly reduced in the presence of oxidizing thiol-reagents such as *o*-iodosobenzoic acid, thus indicating a direct chemical interaction between ergothioneine and these substances.

DISCUSSION

For many years, the evidence for the occurrence of ergothioneine in the animal body rested almost entirely on findings with blood erythrocytes. The discovery of ergothioneine in the seminal vesicle secretion of boar, described in the present study, establishes a second, and incidentally a much richer, source of ergothioneine in the animal body. In addition, it represents the first instance of an extracellular occurrence of this substance in a body fluid of animal origin.

Among the characteristic features of ergothioneine worth noting is the blue colour reaction with Folin's phosphotungstic acid reagent, similar to that given by uric acid, and a high reducing value towards 2:6-dichlorophenolindophenol. The normal oxidation potential of ergothioneine is 0·36 V., as against 0·27 V. for cysteine, 0·32 V. for thiolhistidine, and 0·45 V. for glutathione (Ryklan & Schmidt, 1944). Ergothioneine was shown by Pirie (1933*b*) to share with various substituted thiocarbamides and related substances, the ability to catalyse *in vitro* the oxidation of cysteine and glutathione by hydrogen peroxide.

So far as semen is concerned, it is necessary to remember that observations made on the semen of one animal species do not necessarily apply to other species. There is an analogy to this in the striking morphological differences between the spermatozoa of different species. Thus, the high reducing power of bull semen is largely due to ascorbic acid, of boar semen to ergothioneine, and of semen from ram or

man to a variety of reducing substances. Further complications arise from the variable chemical character of the seminal plasma within one species, and even in the same individual, the chief reason for such behaviour being the peculiar make-up of the seminal plasma as a mixture of secretions from several accessory glands characterized by a fluctuating output (Mann & Lutwak-Mann, 1951).

In boar semen, ergothioneine has been found to originate chiefly in the seminal vesicles, and for this reason the purification and crystallization of seminal ergothioneine was carried out with the boar vesicular secretion as starting material. The chemical identification of the isolated product rests on the results of the elementary analysis, on the preparation of several chemical derivatives, including the crystalline mercury compound, and on the demonstration of all the chief chemical properties required of pure ergothioneine, such as the characteristic content of readily oxidizable sulphur; the reducing power towards a variety of reagents including dichlorophenolindophenol and Folin's 'phosphotungstic acid reagent for uric acid'; and the striking diazo reaction which makes it possible to demonstrate the presence of ergothioneine in a few drops of the boar vesicular secretion.

Work on the biogenesis of seminal ergothioneine is now in progress, but three facts which may be relevant have already been established, namely the absence of thiolhistidine, either free or bound, in the vesicular secretion and in the seminal vesicle tissue; the absence of thiocyanate; the absence of inorganic sulphate. In fact, ergothioneine accounts for nearly all of the non-protein sulphur in the boar vesicular secretion. Future investigations will no doubt answer the question as to the endogenous or exogenous origin of ergothioneine in boar semen. Similar issues are, of course, involved in the problem of ergothioneine occurrence in the blood. Of all mammals, pig has the highest concentration of ergothioneine in the blood. On the basis of nutritional studies it has been claimed that blood ergothioneine is exogenous (Eagles & Vars, 1928; Potter & Franke, 1935); variations in blood ergothioneine according to geographical location have been observed by Hunter (1951). An increased level of ergothioneine in the blood of diabetics has been reported by Benedict *et al.* (1926), Salt (1931) and Fraser (1950).

Concerning the possible physiological role of ergothioneine in semen, it is at the moment by no means easy to give an adequate answer. However, the following points merit attention. Boar semen, with its characteristically high content of ergothioneine, differs further from that of other animals by its very large volume and, at the same time, low density of spermatozoa. These properties have a direct bearing on the short survival time and poor 'keeping quality' of boar sperm *in vitro*, and limit at present the value of boar semen in the practice of artificial insemination. Moreover, the period of time required for the completion of a single ejaculation in the boar is much longer than in other animals. In addition, boar semen as a whole, that is sperm and seminal plasma together, is ejaculated directly into the uterus. Possibly all, or some at least, of these circumstances may be involved in the relationship between the high content and the physiological role of ergothioneine in boar semen.

The results of past investigations by Brachet (1944), Barron *et al.* (1949), and MacLeod (1941, 1946) have given an indication of the importance of SH-groups in spermatozoa for their normal motility and metabolism. In his study on human sperm, MacLeod (1951) was able to demonstrate the protective effect of cysteine and glutathione against the Cu^{2+} inhibition of motility and glycolysis. However, a similar protective action of ergothioneine is of considerably greater physiological significance, in view of the actual occurrence of ergothioneine in the seminal plasma. The present study provided evidence that ergothioneine can counteract most effectively the paralysing action of several thiol-reagents including Cu^{2+} and *o*-iodosobenzoic acid, on the metabolism and motility of spermatozoa. Of particular interest is the antagonistic effect of ergothioneine towards hydrogen peroxide, in view of the demonstration by Tosic & Walton (1950) that toxic amounts of hydrogen peroxide are formed under certain conditions by the spermatozoa themselves in the course of their aerobic metabolism. So far, no adequate evidence has been furnished for a specific function of ergothioneine in blood. However, Spicer, Wooley & Kessler (1951) found that the rates of methaemoglobin formation and reduction in nitrite-treated erythrocytes were related inversely to the blood ergothioneine levels. It is conceivable that one at least of the functions of ergothioneine in both semen and blood may be the maintenance of intracellular SH-groups in a physiologically active condition.

SUMMARY

1. Ergothioneine has been found in boar semen. It is a constituent of the seminal plasma and not of the spermatozoa themselves. It is produced in the seminal vesicles, and does not occur in any other accessory gland secretions.

2. Ergothioneine in the boar vesicular secretion accounts for the major part of the total reducing material which reacts with 2:6-dichlorophenolindophenol in the cold, and which yields the blue colour with Folin's 'phosphotungstic acid reagent for uric acid'.

3. The concentration of ergothioneine in the vesicular secretion was determined in samples from

twenty boars. The contents ranged from 29 to 256 mg./100 ml.; the average was 79 mg./100 ml.

4. From 1300 ml. of boar vesicular secretion 0·48 g. pure crystalline ergothioneine has been isolated. The product has been identified by elementary analysis, the determination of the bromine-oxidizable sulphur, diazo reaction, reducing properties, and by the preparation of certain derivatives including the crystalline mercury compound.

5. For the detection of small amounts of ergothioneine in tissues, urine and other body fluids, a chromatographic method has been developed, which depends on the localization of ultraviolet-absorbing areas on paper chromatograms followed by spraying with the diazo reagent or certain other colour-yielding reagents. In this manner it was possible to detect less than 5 μg. ergothioneine, and to distinguish ergothioneine from thiolhistidine, uric acid and ascorbic acid.

6. Thiolhistidine, either free or bound, does not accompany ergothioneine in the boar seminal vesicles or in the vesicular secretion. The content of ascorbic acid in the boar vesicular secretion was low; in bull, on the other hand, there was little ergothioneine, and the high reducing value of the vesicular secretion was largely due to ascorbic acid. Ram and human semen showed the presence of some reducing and diazo-reactive substances which, though not identical, may possibly be related to ergothioneine.

7. Boar urine, unlike boar semen, contained only a negligible amount of ergothioneine. The claim that human urine contains ergothioneine has not been confirmed. No ergothioneine, or only a trace of it, was found in pig foetal fluids, vitreous fluid of the eye, corpora lutea, adrenal glands and thyroid gland.

8. Ergothioneine, in a concentration corresponding to that found in the boar vesicular secretion, protected spermatozoa against the action of sulphydryl inhibitors. Both motility and fructolysis, under anaerobic as well as aerobic conditions, were abolished in sperm suspensions by 10^{-3} M-iodosobenzoic acid, but could be maintained at a normal level if 2×10^{-3} M-ergothioneine was also added. Ergothioneine also counteracted the inhibition caused by cupric ions and hydrogen peroxide.

9. The protective effect of ergothioneine against inhibitors of SH-groups was not limited to sperm but could be demonstrated in muscle glycogenolysis and yeast fermentation.

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Quantitative Paper Chromatography of Reducing Steroids of the Adrenal Cortex

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The methods commonly employed for the determination of steroids furnish only limited information when applied to adrenal extracts. Not only is it impossible to estimate individual members of the group of biologically active corticoids in a mixture, but the presence of many closely related, but inactive, compounds in gland extracts renders the overall determination of reducing or formaldehydogenic activity largely valueless. Since paper chromatography affords a ready means of resolving the complex mixture of cortical steroids into single compounds or groups of closely related compounds, the quantitative evaluation of particular members of the cortical group thus becomes feasible.

It was desired to develop a method which would enable the steroids to be located on the chromatogram and to be determined colorimetrically by elution of the product of the reaction, thus avoiding the necessity of running reference strips or relying on the R_F values for the elution of the appropriate portions. A survey of the known reactions of steroids applicable to paper chromatograms indicated that they either lacked sensitivity or that they were not quantitative. The arsenomolybdate reaction reported previously (Schwarz, 1952*a*) was found to be satisfactory in both respects. The technique described below is simple and does not require any elaborate equipment. It readily lends itself to routine analysis of mixtures of cortical steroids and it should prove useful for the determination of reducing cortical steroids in blood and urine.

EXPERIMENTAL

Chromatography

The solvent systems described by Zaffaroni, Burton & Keutmann (1950) and by Bush (1952) have been used. With the former the more polar and the less polar steroids have to be resolved on separate strips, one being run in toluene/propylene glycol, the other in benzene/formamide. The time required for satisfactory separation depends on a number of factors, but it is reasonably constant under strictly standardized conditions. In the experience of the author, 6–7 hr. development with benzene of the formamide-impregnated strips gives a separation of the fast-running steroids satisfactory for quantitative purposes, while a running time of about 100 hr. in toluene ensures a reasonable separation of hydrocortisone (17-hydroxycorticosterone) from very polar material at the top of the chromatogram.

Several of the solvent systems described by Bush (1952) enable all the biologically active steroids to be separated from each other on one strip and in a much shorter time. The practical difficulties associated with chromatographing at 34° have been overcome by the use of the 'chromatocoil' (Schwarz, 1952*b*), in which the paper strip is accommodated in spiral form in a container which can readily be placed in an incubator. A paper strip 50 cm. in length, or longer, can easily be fitted into an apparatus measuring no more than 5 cm. diameter × 10 cm. long. The use of the ascending technique has the advantage that development stops automatically when the solvent front reaches the end of the strip, thus making accurate timing unnecessary. Moreover, with the same length of strip, the steroids will always travel approximately the same distance. While chromatographic development is somewhat slower than in the descending arrangement, saturation is effected much more quickly owing to the dimensions of the apparatus. Thus a complete run can be carried out in 5–6 hr.

The biologically active steroids separated chromatographically in any of the solvent systems referred to above are still associated on the chromatogram with biologically inactive steroids. Further resolution can be achieved by rechromatographing each group of steroids separately and for longer periods (Zaffaroni & Burton, 1951).

After chromatography the paper strips are air-dried, or, in the case of propylene glycol or formamide-saturated strips, they are placed in a stream of warm air (about 70°) for 15 min. The dry strips are then ready for treatment with arsenomolybdate reagent.

Reagent. This is prepared according to Nelson (1944). A solution of 25 g. ammonium molybdate in 450 ml. water is mixed with 21 ml. conc. H_2SO_4, and a solution of 3 g. $Na_2HAsO_4.7H_2O$ in 25 ml. water is added. The mixture is incubated at 37° for 48 hr. and filtered. It is stored in a glass-stoppered brown bottle.

Application of reagent and incubation

Many different methods of impregnating the paper strips with the reagent were tried, but all of them were found wanting in one or more respects, e.g. uneven wetting of different parts of the strip; adsorption of arsenomolybdate on one edge; loss of steroids; or longitudinal displacement of the steroids. (Spraying was ruled out on account of the nature of the reagent.)

Qualitative method. The following method was found to give the most satisfactory results for qualitative work, in spite of the loss of a small amount of steroid matter.

A length of thin, plain-weave, bleached cotton fabric (25 threads/cm., 0·72 g./100 sq.cm.) is boiled in distilled water for about 1 hr., rinsed and dried. A strip, about 1·2 cm. wide, is cut out and placed on a length of flexible plastic material (a piece of washed X-ray film was found suitable) wrapped round a glass plate. Sufficient reagent is then run along the length of the fabric from a teat pipette to form a continuous film. The chromatogram strip is placed on another glass plate, and the wet fabric supported by the X-ray film and glass plate is inverted over it quickly and evenly, without exerting undue pressure. After removing the top glass plate, the X-ray film can be bent backwards, thus enabling the fabric to be separated from the paper strip. By this means the test strip is moistened evenly and simultaneously along the whole length. A ring of Whatman no. 3 filter paper, having a short 'tail' at either end, is then placed around the test strip. The paper ring is kept damp by allowing the tails to dip into water during the incubation. After placing a warm glass plate over the test strip and paper ring, the whole arrangement is incubated on a heavy metal base in an electric oven at 70° for 30–60 min. About 1–2 μg. of deoxycorticosterone (DOC)/sq.cm. can be detected by this method, which is thus considerably more sensitive than other methods commonly employed.

Quantitative method. For quantitative purposes the above method has to be modified slightly, since a small amount of steroid matter diffuses on to the fabric and is thus lost. The procedure adopted is as follows.

The chromatogram strip is covered by another paper strip of the same length and width and the two strips are placed on a glass plate. Two layers of fabric are used in order to increase the volume of reagent. Flooding of the fabric is to be avoided. After inverting the damp fabric over the double paper strips, a film of reagent should be formed between the test strip and the supporting glass plate, and between the two paper strips. The plate supporting the X-ray film is carefully removed, leaving the latter in position. The fabric can then be separated easily from the paper strips as shown in Fig. 1. After surrounding the strips by a paper ring and covering them with a glass plate (previously warmed to 76–77°) the double plates are placed on the metal base heated to the same temperature, and incubated at 70° for 60 min. It is important to keep the surrounding paper ring damp during the incubation, as concentration of the reagent results in high blank readings.

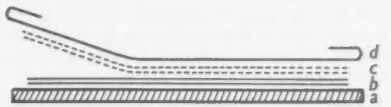

Fig. 1. Diagrammatic representation of arrangement for application of reagent: *a*, glass plate; *b*, test strip and cover strip; *c*, double layer of fabric; *d*, X-ray film. The glass plate supporting the X-ray film has been removed to permit bending of the X-ray film for easier separation of fabric from paper strip.

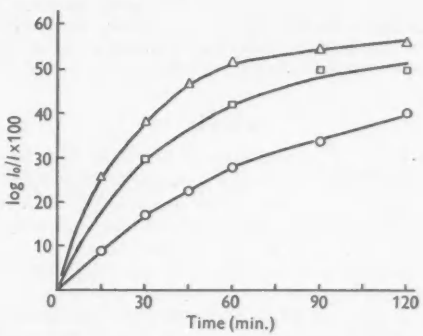

Fig. 2. Rate of reaction at different incubation temperatures. ○, 55°; □, 65°; △, 70°.

Incubation temperature. The temperature of incubation is chosen so that the rate of reaction is sufficiently slow after 1 hr. to permit reproducible results to be obtained. Longer incubation periods have been found unsuitable on account of the greater evaporation from the strip, while shorter periods at a higher temperature increase the uncertainty associated with the raising of the temperature of the cold glass plate and strips to the desired level. Fig. 2 shows the rate of reduction at three different temperatures. It is seen that the reaction is almost complete after 1 hr. at 70°, and this therefore represents the optimum temperature which ensures both accuracy and sensitivity.

Colorimetry

At the end of the incubation the cover plate is removed, the damp strips are laid side by side and appropriate lengths are severed (contact with metallic objects must be avoided) for elution. Lengths of 2·5–5 cm. have usually been found suitable. A similar length is taken from above the starting line to act as a blank. The corresponding lengths are superimposed and eluted together, one end of each pair being

allowed to dip into a shallow Perspex trough, while the other hangs into a colorimeter tube. Alternatively, the top end can be held between two horizontal microscope slides. The molybdenum blue complex is eluted with distilled water and the eluate is made up to a definite volume (4–5 ml.). The optical density is read in a photoelectric colorimeter at 650–660 mμ. against the eluate of the blank strip.

The comparatively high optical absorption of the latter (equivalent to about 2 μg. of DOC/sq.cm.) is not due to the reagent or to impurities in the paper, but results from the partial hydrolysis of the cellulose. The blank is, however, constant over the whole length of the chromatogram strip and is proportional to the length of paper eluted. Thus the appropriate allowance can readily be made for test strips which are longer or shorter than the blank strip.

The blue colour is stable over long periods, but since the reaction is not quite complete after 1 hr. incubation, there is a tendency for the optical absorption to increase slightly on keeping for several hours.

If the amount of reducing material is greater than about 30 μg. of DOC/sq.cm. of strip, a small fraction of the molybdenum blue complex remains adsorbed on the cellulose and cannot be eluted with the volume of water used. In the experience of the author, this is not a serious limitation as such high concentrations are not normally encountered. However, the fraction adsorbed is reasonably constant and allowance can be made for it by running suitable standards.

RESULTS

Solutions of molybdenum blue obey the Beer-Lambert law, but since, in practice, there is a slight deviation from the linear relationship between reducing material present on the strip and molybdenum blue recovered, a standard curve has to be plotted. Moreover, since the reducing power is different for each steroid, separate curves have to be constructed for all corticoids to be estimated, or conversion factors have to be used. The latter procedure will be found sufficiently accurate for most purposes.

Table 1. *Relative reducing power of different steroids*

(60 min. incubation. DOC = 1·0.)

	Arseno-molybdate	Phospho-molybdate
DOC	1·0	1·0
Compound A	0·87	0·84
Cortisone	—	0·74
Hydrocortisone	0·69	—
Methyltestosterone	0·43	0·43
Progesterone	0·33	0·30
Pregn-4-ene-20β:21-diol-3:11-dione	0·17	—
17-Ethinylandrost-4-en-17α-ol-3-one	—	0·17

In Table 1 the relative reducing powers of several steroids are compared with the values obtained by Heard & Sobel using the phosphomolybdate method (Heard & Sobel, 1946).

The agreement between the two sets of values suggests that the various functional groups of the steroid molecule make the same fractional contribution to the total reducing power in both methods. It can therefore be assumed that the same general considerations govern the reducing activity of steroids under the milder conditions of the arsenomolybdate technique as have been found by Heard & Sobel to obtain in the phosphomolybdate method.

The slight deviation from the linear relationship between reducing matter present and molybdenum blue formed may be due to local insufficiency or lack of accessibility of reagent, resulting from the limited diffusion possible under the experimental conditions. Accessibility, in particular, may be restricted in the case of hydrophobic steroids like DOC. It is likely to be aggravated by irregular distribution of the steroid which is difficult to avoid when comparatively large volumes of solution are applied to a restricted area by repeated evaporation.

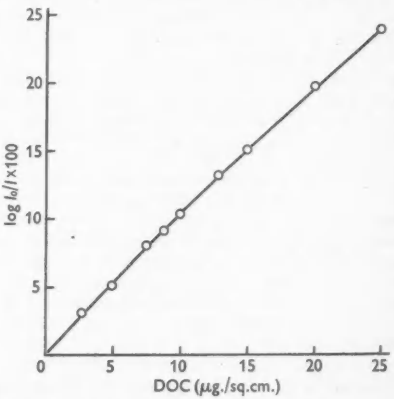

Fig. 3. Reference curve for deoxycorticosterone.

Fig. 3 shows the experimental relationship between the amounts of DOC placed on the strip and the optical densities of the eluates. The average of at least three determinations has been used for the plotting of each point. In no case do individual determinations differ by more than 11 % from the mean, and the average deviation of forty-six determinations is 3·8 %.

As has been shown above, the recoveries fall off as the concentration of DOC/unit area increases. The optical densities have therefore been plotted against concentration/sq.cm. rather than against the total amount of DOC. In using the standard graph it is thus necessary to divide the optical density by the length of the spot (if strips of unit width are used), and to multiply the equivalent amount of DOC by the same value. Since the deviation from a straight

line is small, the spot length need not be determined very accurately.

The method outlined above has been applied to the quantitative analysis of two synthetic mixtures of steroids. Table 2 shows the mean of the determinations, the standard deviation, and the recovery.

Of the many adrenal extracts examined, analytical results obtained with two experimental extracts are given in Table 3.

value of the paper strip itself, it is, however, not possible to determine less than 5 μg. of steroid with any accuracy. The upper limit is set by the local availability of reagent and the adsorption of the blue complex on to the paper. With an average elongation of the steroid spot during chromatography to 2–3 cm., up to 100 μg. can be dealt with satisfactorily. This represents a range of usefulness sufficiently wide for most purposes.

Table 2. *Analysis of synthetic mixtures*

Mixture I

Compound	Present (μg./ml.)	Found (mean of four determinations) (μg./ml.)	S.D.	Recovery (%)
F	10	9·75	0·75	98
E	8·8	8·85	0·58	101
B	20*	16·25	1·27	—
A	20	17·20	0·38	86

Mixture II

Compound	Present (μg./ml.)	Found (mean of three determinations) (μg./ml.)	S.D.	Recovery (%)
F	20	19·87	1·16	99
E	26·4	25·63	0·29	97
B	13*	11·00	0·93	—
A	40	37·47	1·14	94
DOC	20	17·13	0·42	86

* Compound B was not available in crystalline form and was prepared from adrenal extract by paper chromatography. The absolute amount of B present in the synthetic mixtures is therefore not known accurately.

Table 3. *Analysis of adrenal extracts*

Steroids associated with	Extract *A* (mean of three determinations) (μg./ml.)	S.D.	Extract *B* (mean of two determinations) (μg./ml.)	S.D.	R_F	*A*/*B*
Compound F	40·2	0·32	33·35	0·78	0·04	1·2
Compound E	23·8	1·11	17·35	0·64	0·12	1·4
Unidentified steroid	13·4	0·87	8·2	0·28	0·29	1·6
Compound B	23·4	1·97	12·45	0·50	0·42	1·9
Compound A	26·0	1·03	13·1	0·85	0·54	2·0

Extract *A* is a purified solution of cortical steroids in ethyl acetate; extract *B* was prepared from *A* by distilling off the organic solvent in the presence of normal saline and filtering off the precipitate.

The loss of steroid material due to removal of the precipitate would be expected to increase with the diminishing hydrophilic nature of the steroid. This is fully borne out by the experimental results and is shown by the ratio *A*/*B* in the last column of Table 3. The increasing R_F values (in toluene-light petroleum/aqueous methanol) are a measure of the decreasing solubility in water.

DISCUSSION

The arsenomolybdate reaction is very sensitive, 1–2 μg. of reducing steroid per sq.cm. being readily detected. In view of the comparatively large blank

It is apparent from the standard deviations presented in Tables 2 and 3 that agreement between repeated analyses is reasonably good. All individual results obtained are, in fact, within less than 10 % of the mean values. In the analysis of the synthetic mixtures the amounts of steroids recovered are, on the whole, satisfactory, although recovery tends to be somewhat lower in the case of the faster-travelling steroids.

The principal weakness of the method lies in the comparatively large optical absorption of the blank strips. Many attempts have been made to increase the accuracy by reducing the blank value, but all have been unsuccessful. Thus, no benefit can be derived from a shortening of the incubation time or a change in the temperature. While the absolute value of the blank can be reduced considerably, it remains substantially the same in relation to the

reducing power of steroids. The low pH of the reagent, which is responsible for hydrolysis of the cellulose, cannot be raised materially without seriously affecting the sensitivity. The latter depends on the formation of the yellow arsenomolybdate complex which requires a large excess of acid.

In the experience of the author, variations of the blank value and serious disagreement between duplicate determinations are largely due to contamination of the chromatogram strips with extraneous reducing matter. It is therefore apparent that a minimum of handling of the paper strips and scrupulous cleanliness on the bench and in the laboratory atmosphere are essential for satisfactory operation.

In spite of its inherent weaknesses, the arsenomolybdate method is capable of furnishing valuable information on the composition of adrenal extracts. It is hoped eventually to correlate the results obtained by quantitative chromatography with those of biological assays, thus enabling an estimate to be made of the potency, as well as the steroid distribution, of a particular extract.

SUMMARY

1. A method is presented for the quantitative evaluation of adrenal extracts in which the mixture of cortical steroids is resolved into its components by paper chromatography and the chromatogram is incubated with arsenomolybdate reagent. Steroids containing a ketol side chain or having an $\alpha\beta$-unsaturated 3-keto structure quantitatively reduce the reagent to molybdenum blue.

2. The blue zones of the developed chromatogram corresponding to the various cortical steroids are eluted and the blue complex is determined in a photoelectric colorimeter.

3. About 5–100 μg. of reducing steroid can be determined with an accuracy of $\pm 10\%$.

4. The reducing steroids are determined in two synthetic mixtures and two adrenal extracts.

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Studies in Rhodopsin

6. REGENERATION OF RHODOPSIN

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The history of visual pigments has been reviewed on numerous occasions, e.g. Collins & Morton (1950) and Wald (1951). Under the action of light the rhodopsin content of the eye decreases to an extent depending on time and intensity, whereas in darkness it gradually approaches a maximum. The photochemical destruction ('bleaching') is, however, normally incomplete (Lythgoe, 1940) as rhodopsin is found in light-adapted eyes. The regeneration of rhodopsin, in terms of dark adaptation in the living eye, approaches completion in 30 min. and is almost complete in 45 min. Both vitamin A deficiency (Hecht, Hendley, Frank & Haig, 1946; Medical Research Council, 1949) and anoxia (Hecht & Mandelbaum, 1940) are known to increase the time necessary for dark-adaptation.

Collins (1951) submitted a brief communication to the Biochemical Society reporting work on regeneration of rhodopsin. These experiments used as starting points those of Zewi (1939) in his important quantitative extension of the pioneer work of Kühne (1878). In the present work *in vitro* preparations of eye tissues have been substituted for excised frog eyes, with exposed optic cups, as used by Zewi. Regeneration of rhodopsin might be either an endergonic or exergonic process. If the latter were the case the reaction would be spontaneous, while if the former were true it would have to be coupled to

an exergonic reaction. It was felt that the best results would be obtained by assuming the more complex alternative, and, if regeneration were achieved, the conditions could then be simplified until the minimal requirements were known. Retinas (or retinas plus choroids) from either frogs or rats were ground up and suspended in a 'complete' medium, i.e. one capable of maintaining oxidative phosphorylation. In this way it was hoped to favour the coupling of exergonic systems to the assumed endergonic regeneration. The capacity for regeneration of rhodopsin after bleaching was ascertained, and various components of the medium were then withheld one at a time.

EXPERIMENTAL

Materials

'Complete' medium. Table 1 shows the composition of the medium used. This is very similar to one used by Potter (1945) except that vitamin A has been included.

Vitamin A solution. Crystalline synthetic vitamin A alcohol (Hoffmann La Roche) was added to a solution of Dispersol A (Imperial Chemical Industries Ltd.) or Tween 80 (Honeywell and Stein Ltd., 21 St James's Square, S.W. 1). (*a*) Dispersol A: a few mg. of the vitamin were added to water (5–10 ml.) and one drop of detergent. The mixture was shaken at 50°, until the vitamin was thoroughly dispersed. (*b*) Tween 80: the detergent was dissolved in $CHCl_3$ and the solution added to a few mg. of vitamin A. When the vitamin had dissolved, $CHCl_3$ was removed under reduced pressure and the desired volume of water was added (cf. Bliss, 1951).

To determine the concentration of vitamin A in such aqueous dispersions a small portion was diluted with a known volume of ethanol to bring the intensity of absorption at 320–330 mμ. within the range of the photoelectric spectrophotometer. The absorption curve in the region 310–340 mμ. was plotted and $E_{max.}$ corrected where necessary for irrelevant absorption (Cama, Collins & Morton, 1951).

Adenosinetriphosphate (ATP). This was purchased either as the barium salt (Boots Pure Drug Co., Ltd.) or as the sodium salt (L. Light and Co., Ltd.). The barium salt was converted to the potassium salt after precipitation of $BaSO_4$ and neutralization of the acid with KOH (Bailey, 1949).

Diphosphopyridine nucleotide (DPN). This was supplied by the Schwarz Laboratories (New York).

Cytochrome c. This was obtained from Evans Medical Supplies, Ltd. It was dialysed before use and standardized as described by Potter (1945).

Digitonin solution. The solution (1% w/v in water) was prepared by boiling the requisite amount of digitonin with water and cooling as soon as it had become clear.

Buffer solution (pH 9·3). This consisted of 19 g./l. of $Na_2B_4O_7 \cdot 10H_2O$.

Potash alum. $K_2SO_4 \cdot Al(SO_4)_3 \cdot 24H_2O$, 4% (w/v) in water.

Methods

Animals. Eye tissues were obtained from frogs (*Rana esculenta*) and from rats, both albino and piebald.

Dissection. To dissect the eyes an incision was made close to the junction of the retina and conjunctiva. The incision was then continued with a pair of scissors all around the ora terminalis, thus enabling the cornea, lens, aqueous humour and vitreous humour to be removed. Using a pair of curved forceps the retina, choroid and pigment epithelium were then scraped out and transferred to a centrifuge tube.

Procedure. At least five animals were needed for one experiment, five right eyes serving as a control to the five left eyes. After dissection 0·5 ml. of the medium was added to each lot in a centrifuge tube and the mixture bleached for 1 min. with a photoflood lamp. The retinas, choroids and pigment epithelia were then ground up with a glass rod and finally incubated in the dark for 2 hr. In the case of the frog tissue, moist air was blown into the mixture kept at room temperature. This agitated and aerated the suspension. Moist air and, in a later experiment, moist N_2 were both obtained by bubbling the gas through water, thus preventing the suspension from going dry. With rat tissues, the tubes containing the retina, choroids and medium were stoppered and shaken in a bath kept at 37°. All experiments were done in pairs: e.g. (1) unbleached vs. bleached and then regenerated, (2) bleached and regenerated vs. bleached and not incubated, and (3) bleached and regenerated with vs. without ATP.

Extraction and determination of rhodopsin. Alum solution (4%, w/v) was added to each tube and the mixture left to stand in the dark for 1 hr. It was then centrifuged, the supernatant liquor was discarded and the precipitate washed once with saline solution (0·9%, w/v, NaCl in water). With frog tissues the digitonin solution (usually 0·5 ml.) was then added and left for 1 hr. at room temperature. The solution was again centrifuged and the supernatant liquor poured off and mixed with an equal volume of buffer solution (pH 9·3). This mixture was recentrifuged before being placed in the cell for spectrophotometric examination at 500 mμ. or, occasionally, over the range 400–600 mμ. With rats, usually 1 ml. of digitonin solution was used for the extraction of rhodopsin. No borate buffer was added since it caused precipitation of some of the rhodopsin. The mixture was then centrifuged and the digitonin extract examined as for frogs. The solution was then 'bleached' by exposure to intense white light and the absorption remeasured. The difference in the intensities of absorption at 500 mμ. was taken as a measure of the rhodopsin content. A 1 cm. cell was used throughout. When the volume of rhodopsin solution was less than 2 ml. the cell was modified. Two pieces of thin glass cut to fit inside the cell were placed on either side of the light path. In this way the volume of liquid necessary was reduced without interfering with the light falling on the photo-cell. The compensating cell, in all cases, contained distilled water, since only the difference spectrum was important. The yield of rhodopsin as recorded in this paper means:

$$\frac{\text{(Extinction at 500 m}\mu\text{. due to rhodopsin)} \times \text{(volume of soln)}}{\text{(Number of retinas)}}.$$

RESULTS

Experiments with frogs

Using the complete medium (Table 1) regeneration with frog eye tissue could be demonstrated. Experiments were then carried out to determine the necessity or otherwise of components of the

medium for optimal regeneration and the results are shown in Table 1. The figures given are averages of a number of experiments. Both vitamin A and ATP were necessary; in the absence of either the yield fell to about 50 %.

Table 1. *The results obtained from a series of experiments on the regeneration of rhodopsin using frog retinas and choroids*

(Reaction mixtures of 1 ml. were prepared and used as described in the experimental section. Final concentrations of reactants were: potassium succinate, 0·02 M; phosphate buffer, pH 7·4, 0·034 M; magnesium sulphate, 0·005 M; nicotinamide, 0·03 M; cytochrome *c*, 1×10^{-5} M; vitamin A alcohol, $2–8 \times 10^{-4}$ M; diphosphopyridine nucleotide, 5×10^{-5} M; adenosinetriphosphate (ATP), $1·3 \times 10^{-3}$ M).

	Yield per eye (fiducial limits for $P = 0·05$) (for units see text)	No. of samples	Percentage yield
Unbleached	0·036 ± 0·0037	8	100
Bleached	0·008 ± 0·0022	8	22
Regenerated with complete medium	0·033 ± 0·0033	8	92
ATP not added	0·016 ± 0·0053	3	44
Vitamin A not added	0·018 ± 0·0076	5	50

Table 2. *Regeneration of frog rhodopsin under varying conditions; individual experiments*

(The reaction mixture used was the same as in Table 1 except where indicated.)

	Yield per eye (for units see text)	Percentage yield
—	0·029	100
Nicotinamide and diphosphopyridine nucleotide withheld	0·016	55
—	0·028	100
Cytochrome *c* withheld	0·016	57
—	0·031	100
N_2 used instead of air	0·022	71
Regenerated for 1 hr.	0·026	79
Regenerated for 2 hr.	0·033	100
Time between killing and starting the experiment:		
(*a*) 40 min.	0·027	100
(*b*) 120 min.	0·019	70

A number of further experiments were carried out (Table 2). (i) When either cytochrome *c* or DPN plus nicotinamide was omitted the yield fell to about 55 % of the control in which the complete medium was used. (ii) If moist nitrogen was blown into the mixture instead of air the regeneration fell to 71 %. (iii) The supply of frogs was not large enough to enable a complete regeneration-time curve to be constructed. In one experiment the regeneration after 1 hr. was 97 % of that after 2 hr. (iv) The final experiments illustrate the fact that the amount of regeneration decreases if the eye tissues are kept too long before incubation even if the complete medium is used. Potassium succinate was not needed in the medium.

Experiments with rats

At first, albino rats were used, but it was soon apparent that the amount of rhodopsin in unbleached retinas was low. Regeneration in these experiments was so small as to be negligible. The average yield of rhodopsin for thirty-five albino rat eyes was 0·009, compared to 0·024 for piebald rats. After weaning, eight animals of both types were fed on the standard diet used in the small-animal colony in this laboratory. The amount of vitamin A in the livers was between 600–700 i.u./g. in both cases. The yield of rhodopsin from unbleached eyes of albino rats was 0·008; the corresponding figure for the piebald rats was 0·027. After an initial bleaching followed by incubation at 37° for 2 hr. in the dark, using the 'complete' medium, the yield for the albinos was 0·0025 while the piebald rats gave a yield of 0·017. After this experience only piebald rats were used.

A number of experiments were carried out in which the need for each component of the 'complete' medium was tested. It was found that maximal regeneration could be obtained when the medium contained only phosphate buffer, magnesium ions and vitamin A. The need for magnesium ions was not investigated.

The yield of rhodopsin in twenty-five regeneration experiments was 0·0179 ± 0·0008. The experimental conditions are quite critical. A large number of other experiments failed to give regeneration in that the results did not differ significantly from the yield at zero time, and a combined figure of 0·0075 ± 0·0006 (thirty experiments) was obtained. This figure is not easy to interpret, although various tentative explanations could be offered. From the present point of view it offers a convenient base line for assessing the extent of regeneration. The average yield for the fully dark-adapted eye, using the figures obtained by Collins & Morton (1950) in conjunction with those obtained in ten experiments in the present work, was 0·0236 ± 0·006. The average amount of regenerated rhodopsin is thus 76 % of the rhodopsin content of the dark-adapted living rat eye.

Terner, Eggleston & Krebs (1950) showed that oxidative phosphorylation was possible with cattle retinas in the presence of D-glucose and L-glutamic acid. Adapting their experimental procedure, the left eyes of six rats were dissected on ice and incubated for 2 hr. with a medium containing only phosphate buffer, glucose and glutamic acid. The six right eyes were dissected at room temperature and incubated with the same medium for the same length of time as a control. The results of this

Table 3. *Experiments on the regeneration of rhodopsin showing the effect of dissection at room temperature and at 0°, and the effect of vitamin A and of 2:4-dinitrophenol*

(Basic medium: 0·04 M-glucose, 0·02 M-glutamic acid, 0·034 M-phosphate buffer (pH 7·4). When used, the concentration of vitamin A alcohol was $2–8 \times 10^{-4}$ M and the 2:4-dinitrophenol was 1×10^{-4} M.)

Conditions of dissection	Change in basic medium	Experimental pair*			
		1	2	3	4
Room temperature	None	0·008	0·012	—	—
0°	None	0·014	0·017	—	—
0°	+ Vitamin A	—	—	0·019	0·020
0°	+ Vitamin A and 2:4-dinitrophenol	—	—	0·014	0·017

* The experimental pairs are entered in the same column.

experiment are shown in Table 3, from which it is apparent that the best regeneration was obtained when the operations, prior to bleaching and incubation, were carried out at a temperature near 0°.

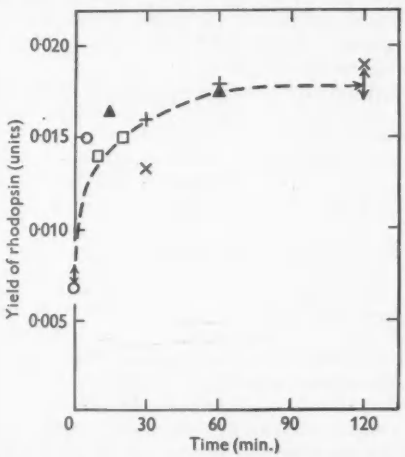

Fig. 1. Relationship between the amount of regeneration using rat retinas and choroids dissected out at room temperature, and the time of incubation in the dark using a medium containing $2–8 \times 10^{-4}$ M-vitamin A alcohol and 0·034 M-phosphate buffer (pH 7·4). The average ranges obtained in a number of experiments, for the amount of rhodopsin at zero time and after 2 hr. incubation, are shown by vertical lines. The remaining points on the curve were determined in pairs (six right and six left eyes in each experiment) and each pair has the same symbol. For definition of rhodopsin units see text.

The addition of 2:4-dinitrophenol appeared to neutralize the beneficial effects of vitamin A. This effect of vitamin A is in agreement with the observation already made that vitamin A is necessary for a maximum regeneration when the dissection procedure is carried out at room temperature.

Attempts were then made to construct a curve relating the amount of regeneration to time of incubation. As it was impossible to do this in a single experiment it was necessary to construct a composite curve using data from five experiments. Since one could not be certain that the rats in the different experiments were comparable, this may account for the rather large scatter of the points obtained. However, it is quite clear that most of the regeneration is complete in 1 hr. (see Fig. 1).

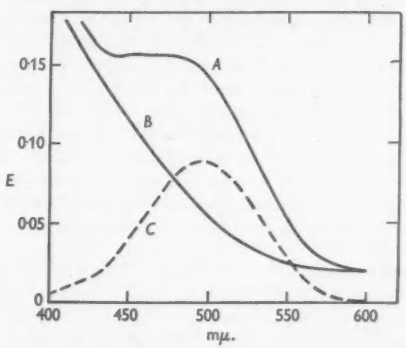

Fig. 2. The absorption spectrum of regenerated rat rhodopsin. Curve *A* is the spectrum of the unbleached solution while curve *B* is the spectrum of the same solution after bleaching. Curve *C* is the difference between *A* and *B* and represents the absorption due to rhodopsin.

Fig. 2 shows the absorption spectrum of a solution of regenerated rhodopsin.

DISCUSSION

Zewi (1939), working with frogs (*Rana esculenta*), completed an extensive analysis of the regeneration of rhodopsin in living animals, and on excised opened eyes. He found that the course of the regeneration in live frogs was influenced by temperature; within the range studied 22·1° was the optimal. In excised open frog eyes no influence of temperature on regeneration was observed. At

7·2° the regeneration proceeded as rapidly in the excised open eyes as in the intact animals at the same temperature. Thus, part of the regeneration in the living frog eye must be susceptible to temperature and this suggests that the regeneration of rhodopsin may follow one of two paths. Further evidence that this is so was obtained by Zewi (1941), who demonstrated that the component of regeneration which is susceptible to temperature is also influenced by drugs. Atropine retarded the process at 22·4°, but had no effect at 8°. This suggests that the energy used for the phase of regeneration sensitive to both temperature and drugs is dependent on living processes. Zewi also demonstrated that the presence of oxygen was necessary for regeneration. Regeneration *in vitro* has been obtained with both species. With frogs, the need for oxygen, vitamin A and a medium capable of supporting oxidative phosphorylation was demonstrated, but rats needed only vitamin A. The retina of the frog differs from the mammalian retina in its dependence on the choroidal rather than the retinal circulation for its nourishment. This difference may be responsible for the difference observed in the medium which will give maximum regeneration in the frog and rat eyes. With rats the necessity for vitamin A has been demonstrated either when the eyes are dissected at room temperature or under ice-cold conditions. The presence of ATP in the medium is not necessary using rat tissue under these latter conditions, but when the rat eyes are dissected at room temperature conflicting results have been obtained; the majority of experiments, however, have been successful in its absence.

The results obtained using 2:4-dinitrophenol make it tempting to draw the conclusion that phosphorylation is necessary for the utilization of vitamin A alcohol. According to the present state of knowledge (Copenhauer & Lardy, 1952) 2:4-dinitrophenol is a more effective inhibitor of phosphorylation coupled with electron transport (reduced diphosphopyridine or triphosphopyridine nucleotide-flavoproteins-cytochromes) than of simple substrate phosphorylation (as in anaerobic glycolysis). In the frog experiments the necessity for ATP in the medium used for a maximum regeneration can be regarded as substantiated. With rats the fact that it is not necessary to add ATP to the medium does not mean that it plays no part either directly or indirectly in the process of regeneration. If an endergonic reaction is concerned in the regeneration of rhodopsin, it is conceivable that the energy required (presumably as ATP) may be obtained from glycolysis.

Wald and his co-workers (Wald & Brown, 1950; Wald & Hubbard, 1950, 1951) have presented evidence concerning the mechanism of rhodopsin synthesis using frogs' eyes (*Rana pipiens*) and cattle eyes. It appears that in all their experiments involving the use of tissue homogenates in the presence of vitamin A the period of incubation used varied from 9 to 12 hr. The present work with frogs and rats has shown that the greater part of the regeneration is over after 1 hr. This agrees closely with the time required for dark-adaptation in the living eye.

The following comments can be made. It is clear from the present work that regeneration of rhodopsin can occur *in vitro* and can be studied in detail. The natural process, which requires vitamin A and is complete in less than an hour, is closely simulated *in vitro*. When eye tissues from rats or frogs are used the experimental work is difficult and tedious, largely because the absolute amounts of rhodopsin are quite small. It is, therefore, a considerable advantage to be able to use cattle retinas not only because they are plentiful but also because they are so much larger.

Wald and his co-workers have published observations on the regeneration of rhodopsin which appear to differ from some of those presented here. Their tentative interpretation also differs, but it is too early to debate this and both experimental approaches need to be continued.

SUMMARY

1. The regeneration of rhodopsin has been studied using isolated retinas with choroids, from frogs (*Rana esculenta*) and rats.

2. With frogs' retinas 92 % regeneration could be obtained using a medium containing vitamin A alcohol, adenosinetriphosphate, diphosphopyridine nucleotide, cytochrome *c*, and nicotinamide. Oxygen was also necessary.

3. Although albino and piebald rats did not differ in respect of the amount of vitamin A stored in the liver, the rhodopsin content of the eyes of piebald rats was more than three times greater than that of albino rats. Moreover, the albinos had extremely low capacity for regenerating rhodopsin.

4. When rat retinas and choroids were dissected out with the minimum delay after death, maximum regeneration (76 %) was obtained using a medium containing only phosphate buffer and vitamin A.

5. The time required for regeneration *in vitro* in rat retinas and choroids is between 30 and 60 min., in good agreement with human dark-adaptation time.

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Spectrophotometric Determination of Fructose-1:6-Diphosphate, Hexosemonophosphates, Adenosinetriphosphate and Adenosinediphosphate

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Spectrophotometric methods are now widely used for following the course of certain enzymic reactions. When the reverse reaction is slight, it is possible to adapt the procedure so that it becomes a specific and usually very sensitive method of measuring the concentration of the reactants. Reactions involving oxidized or reduced diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) have been particularly useful, since the reduced coenzyme absorbs strongly in the near ultraviolet (Warburg & Christian, 1936) and a number of enzymes catalysing the reaction between various intermediary metabolites and the coenzyme can be prepared. Examples are the estimation of pyruvate and malate (Ochoa, Mehler & Kornberg, 1948), *iso*-citrate (Ochoa, 1948), α-ketoglutarate (Kornberg & Pricer, 1951*a*) and glucose-6-phosphate (Ochoa, Salles & Ortiz, 1950; Slein, 1950; Kornberg & Pricer, 1951*b*).

In connexion with investigations of oxidative phosphorylation, a very sensitive method of determining fructose-1:6-diphosphate (HDP) was required. Dr Racker suggested to me that his method (Racker, 1947) of measuring phosphohexokinase activity might be adapted for this purpose. The present paper describes the successful adaptation of this method for the estimation not only of HDP, but also of the hexosemonophosphates (glucose-6-phosphate, fructose-6-phosphate and glucose-1-phosphate) and adenosinetriphosphate (ATP) and adenosinediphosphate (ADP). It can also be adapted for the measurement of creatinephosphate. A preliminary account of this work has already been published (Slater, 1951).

PRINCIPLE OF METHODS

Procedure A. In the presence of rabbit-muscle fraction *A* (see Methods), HDP reacts with an excess of reduced DPN, according to the following scheme:

(1) HDP → glyceraldehydephosphate + dihydroxyacetonephosphate (*aldolase*),

(2) Glyceraldehydephosphate → dihydroxyacetonephosphate (*triosephosphate isomerase*),

(3) 2 Dihydroxyacetonephosphate + 2 (reduced DPN) → 2 glycerolphosphate + 2DPN (*glycerolphosphate dehydrogenase*).

Overall reaction (*A*): HDP + 2 (reduced DPN) → 2 glycerolphosphate + 2 DPN.

The rabbit-muscle fraction *A* contains the necessary enzymes shown in brackets after the equations. Since the equilibrium constant of reaction (3) is $1{\cdot}4 \times 10^4$ at 22° and pH 7 (Baranowski, 1949), the overall reaction *A* proceeds virtually to completion in the direction shown and the disappearance of reduced DPN, determined by the decrease of optical density at 340 mμ., is a measure of the HDP concentration. The alternative reaction of glyceraldehydephosphate, namely oxidation by DPN in the presence of inorganic phosphate and glyceraldehydephosphate dehydrogenase, does not occur with the rabbit-muscle fractions used (see below).

In this procedure, and throughout the paper, it should be understood that any glyceraldehydephosphate or dihydroxyacetonephosphate present will be included in the estimation of HDP. For most purposes, the estimation of the sum of HDP and the two triosephosphates is more useful than the estimation of only the HDP. L-Sorbose-1-phosphate, which liberates one molecule of dihydroxyacetonephosphate when treated with aldolase (Lardy, Wiebelhaus & Mann, 1950) would no doubt behave in the same way as triosephosphate.

Procedure B. If Mg^{++}, excess ATP and rabbit-muscle fraction *B* (see Methods) are added to the components used in procedure *A*, the three hexosemonophosphates which appear in glycolysis will react as follows:

(4) Glucose-1-phosphate ⇌ glucose-6-phosphate ⇌ fructose-6-phosphate (*phosphoglucomutase* and *hexosemonophosphate isomerase*),

(5) Fructose-6-phosphate + ATP → HDP + ADP (*phosphohexokinase*),

(6) ADP → ½ ATP + ½ adenylic acid (*myokinase*),

(*A*) HDP + 2 (reduced DPN) → 2 glycerolphosphate + 2 DPN.

Overall reaction (*B*): HMP + ~P + 2 (reduced DPN) → 2 glycerolphosphate + 2 DPN,

where HMP is the sum of the three hexosemonophosphates and ~P represents the reactive energy-rich phosphate groups of ATP and ADP. All the necessary enzymes are present in rabbit-muscle fraction *A* or *B*.

This reaction can be used in two ways: (*a*) in the presence of excess ~P the disappearance of reduced DPN is a measure of the total concentration of the three hexosemonophosphates + HDP; (*b*) in the presence of excess HMP the disappearance of reduced DPN is a measure of the HDP + total concentration of ~P of ATP and ADP.

Procedure C. A separate sample of the solution to be analysed is treated with glucose, Mg^{++} and yeast hexokinase. When reaction (*C*)

(*C*) ATP + glucose → glucose-6-phosphate + ADP (*hexokinase*)

has reached completion, the hexokinase is inactivated by the addition of trichloroacetic acid and removed by centrifugation and HMP estimated on the neutralized supernatant by procedure *B*.

EXPERIMENTAL

Materials

Rabbit-muscle fraction A. This was the preparation described by Racker (1947) as 'glycerophosphate dehydrogenase and aldolase'. Racker's procedure was followed exactly. The $(NH_4)_2SO_4$ paste was stored at −15° and a solution freshly prepared for each day's analyses. The paste was dissolved in ice-cold water and filtered to give a concentration of about 20 mg. protein/ml. The solution was kept in ice. The actual concentration used depended upon the activity of the preparation, which declined on storage (it retained sufficient activity for about 3 months), and on the presence of inhibiting substances, such as trichloroacetate, in the solutions to be analysed. The concentration of enzyme used in the test should be such that the reaction is complete within 10 min., preferably 5 min.

Rabbit-muscle fraction B. This was essentially Racker's (1947) phosphohexokinase preparation. The precipitate obtained between 0·2 and 0·5 saturation with $(NH_4)_2SO_4$ during the preparation of rabbit-muscle fraction *A* was dissolved in about 50 ml. 0·01 M-phosphate buffer, pH 7·6, and dialysed overnight against 0·35 saturated $(NH_4)_2SO_4$, pH 7·6. The precipitate was removed by centrifugation in the cold and the supernatant brought to 0·5 saturation with 0·3 vol. saturated $(NH_4)_2SO_4$, pH 7·6. The precipitate was collected by centrifugation in the cold and suspended in a little 0·01 M-phosphate buffer, pH 7·6. The paste, which was stored at −15°, retained sufficient activity for about 3 months. A fresh solution was prepared for each day's analyses by diluting tenfold with ice-cold 0·025 M-glycylglycine buffer, pH 7·6. This solution is faintly cloudy, but does not need filtering.

Hexokinase. Two preparations of yeast hexokinase were used in this study: (*a*) purified hexokinase prepared by fractionation with ethanol and adsorption on alumina gel according to Berger, Slein, Colowick & Cori (1946); (*b*) a crude preparation prepared as follows.

Baker's yeast (6·4 kg.) was autolysed according to the procedure of Allfrey & King (1950). The only modification of this procedure was to make the suspension 1 % with respect to glucose immediately after autolysis (cf. Berger *et al.* 1946). The lower aqueous layer was filtered through a pad of Super-Cel on a Büchner funnel. The filtrate was brought to 0·55 saturation by the slow addition of 350 g. $(NH_4)_2SO_4$/l. and the mixture filtered through large fluted papers overnight. The filtrate was brought to 0·75 saturation by the slow addition of 138 g. $(NH_4)_2SO_4$/l. and the mixture filtered overnight. The precipitate was dissolved in water containing 10 g. glucose, brought to pH 7·0 and diluted to 1 l. The solution was brought to 0·55 saturation by the slow addition of $(NH_4)_2SO_4$, allowance being made for the $(NH_4)_2SO_4$ in the precipitate. The pH was kept at 7·0 by the occasional addition of N-KOH. The precipitate was removed by filtration overnight, the filtrate brought to 0·75 saturation by the slow addition of 138 g. $(NH_4)_2SO_4$/l. and the precipitate collected by centrifugation. It was transferred with a solution containing 0·01 M-acetate buffer (pH 5·4)—1 % glucose

to a dialysis sac and dialysed against this solution until free from sulphate. The dialysed solution was stored at −15°.

A small yield of hexokinase of higher specific activity can be obtained by refractionation of this solution between 0·85 and 0·95 saturation with $(NH_4)_2SO_4$ (Holton, 1952).

Creatinephosphokinase. A preparation from rabbit muscle was kindly supplied by Dr B. Askonas.

Lactic dehydrogenase. This was purified by fractionation of rabbit-muscle fraction *A* with $(NH_4)_2SO_4$, according to the method of Korkes, Del Campillo, Gunsalus & Ochoa (1951). Lactic dehydrogenase crystallized at 0·55 saturation with $(NH_4)_2SO_4$ after standing for several days at 0°. The crystalline precipitate was collected by centrifugation, suspended in a little 0·01 M-phosphate, pH 7·6, and stored at −15°.

DPN. Several preparations of DPN ranging from 33 to 79% purity have been used in this study. Laboratory-prepared samples from yeast by an unpublished method of Ochoa, from liver by the method of LePage & Mueller (1949), and commercially available DPN are all suitable for the estimation of HDP, HMP or ATP. Especially purified DPN is, however, necessary for the estimation of ~P, since many impure preparations contain considerable amounts of ~P. A preparation 79% pure obtained by LePage & Mueller's method was particularly suitable. Dr R. K. Morton (private communication) has found that treatment of DPN, prepared by Ochoa's method, for 4 min. at pH 1·5 and 100° reduces the ~P content to a level suitable for estimations of creatinephosphate.

Reduced DPN. This was prepared either by reduction with $Na_2S_2O_3$ according to Ohlmeyer's (1938) procedure or by reduction with alcohol and alcohol dehydrogenase (Racker, 1950; Bonnichsen, 1950). The following procedure has been found satisfactory.

A sample containing 15 mg. DPN is dissolved in 20 ml. water, 3 ml. 10 M-ethanol (aldehyde-free) added and the solution brought to pH 9·0 with N-KOH and 0·1 N-KOH. Approx. 1 mg. of crystalline yeast alcohol dehydrogenase (Racker, 1950) is added and the pH again adjusted. The solution is diluted to 30 ml. and the optical density at 340 mμ. followed in a 0·5 cm. cell keeping the pH at 9·0. When the optical density has reached a maximum (about 10 min.), the solution is immersed in a boiling-water bath for 5 min., cooled and filtered. The solution can be stored for approx. 2 weeks at −15°. Pure liver alcohol dehydrogenase (kindly supplied by Dr R. K. Bonnichsen) was also used satisfactorily. Since this enzyme has a lower turnover number than that from yeast, a somewhat longer time is required to obtain the maximum reading.

If the DPN sample is contaminated with heavy metals, a little 'Versene' (ethylenediamine tetraacetic acid) should be added before the alcohol dehydrogenase.

ATP. The barium salt of ATP was either obtained commercially or prepared in the laboratory from rabbit muscle by the procedure described by LePage (1949*a*), omitting the magnesium anaesthesia. The mercury precipitation was repeated as described therein. The barium salt was converted into a neutral solution of the potassium salt by passage through an ion-exchange column (Polis & Meyerhof, 1947; Rowles & Stocken, 1950). Following the advice of Dr S. M. Partridge, Dowex 50 was the resin used. This resin effected some purification of the commercial ATP by removing some of the adenylic acid. The procedure adopted was to treat the column with 2 N-HCl and then wash through with water until the eluate no longer turned Congo red paper blue. The barium ATP was dissolved in the minimum volume of cold N-HCl and, after filtration if necessary, poured through the column. The rate of flow (area approx. 1 sq.cm.) was 15 ml./hr. The ATP solution was followed by water. Collection of the eluate commenced when a drop turned Congo red paper blue and ceased when it no longer turned the colour. Alternatively, the optical density at 260 mμ. of the eluate was followed. The eluate was kept cold and finally neutralized with N-KOH. This solution is stable for several months at −15° (cf. Bailey, 1949).

ADP. This was prepared from ATP by the addition of glucose and hexokinase, following a procedure essentially the same as that used by Colowick & Kalckar (1943). The barium salt was isolated and converted into the potassium salt in the same way as for ATP.

Creatinephosphate. A sample of the crystalline sodium salt, prepared by the method of Ennor & Stocken (1948), was kindly supplied by Dr A. Narayanaswami.

Fructose-6-phosphate. A solution of the potassium salt was prepared from the commercial barium salt by means of the ion-exchange column procedure, as described for ATP. This solution contains very little inorganic phosphate.

Glucose-6-phosphate. A sample of the barium salt, prepared synthetically, was kindly supplied by Dr E. Racker. The solution of the potassium salt was prepared by treatment of the barium salt with K_2SO_4. There was no measurable inorganic phosphate in this solution.

Hexosemonophosphate. The supernatant obtained after precipitation of the barium salt of ADP from the reaction mixture after treatment of ATP with hexokinase and glucose (see above) was brought to pH 8·2 and treated with 4 vol. 95% (v/v) ethanol. After cooling in ice, the precipitate was collected by centrifugation and washed with 95% ethanol, followed by ether. The solution of the potassium salt was prepared by treatment of the barium salt with K_2SO_4. The molar concentrations of inorganic phosphate and ADP in this solution were respectively 0·07 and 0·24% that of the hexosemonophosphate. Since the hexokinase used in this preparation very likely contained hexosemonophosphate isomerase, this preparation is probably a mixture of glucose-6-phosphate and fructose-6-phosphate.

Glucose-1-phosphate. A sample of the dipotassium salt (dihydrate) was kindly supplied by Dr D. M. Needham.

Phosphoglyceric acid. A sample of the monobarium salt, kindly supplied by Dr S. Ratner, was treated with K_2SO_4.

Fructose-1-phosphate. A sample of the barium salt, kindly supplied by Dr R. K. Morton, was treated with K_2SO_4.

Phosphopyruvic acid. A solution was kindly supplied by Dr R. K. Morton.

Fructose-1:6-diphosphate. A commercial sample of the barium salt was brought into solution with HCl and treated with K_2SO_4. The solution contained much inorganic phosphate (25% of the organic phosphorus).

Analytical methods

Inorganic P content of acid-labile compounds. This was determined by the method of Berenblum & Chain (1938).

Total P. This was determined by (*a*) digestion with 10 N-H_2SO_4 (LePage, 1949*b*) followed by determination of inorganic P by the method of Lohmann & Jendrassik (1926), or (*b*) incubation with highly purified phosphomonoesterase (kindly supplied by Dr R. K. Morton). At the end of the incubation, the inorganic P was determined by the method of Lohmann & Jendrassik (1926), and the residual phosphorylated sugar was determined by the method described

in this paper. The values for the organic P have been corrected for the small amount of unhydrolysed ester (amounting to 0·5–2·5% of the total).

Acid-labile P. This was determined after treatment with N-HCl for 10 min. at 100°.

Total fructosephosphate. This was determined colorimetrically by Roe's (1934) method, using fructose as standard, on the assumption that fructosemonophosphate gives 79% of the colour given by an equivalent amount of fructose under these conditions (Slein, 1950).

Total adenosine. This was determined by measuring the optical density at 260 mμ. at neutral pH and converting to concentration by means of the value of $1{\cdot}59 \times 10^4$ obtained for the molar extinction coefficient of adenylic acid and ATP by Kalckar (1947).

Description of method

The description which follows is for the separate estimation of HDP, HMP, ATP and ADP in a mixture, assuming that no interfering materials are present. Methods of overcoming certain interfering substances will be described later. Separate portions of the reaction mixture are subjected to procedures *A*, *B* (*a*), *B* (*b*) and *C*.

Procedure A. Three spectrophotometer cells (1 cm.) are filled as follows:

	1 (ml.)	2 (ml.)	3 (ml.)
Glycylglycine, 0·25 M, pH 7·6	0·3	0·3	0·3
Reduced DPN, 8×10^{-4} M approx.	—	0·3	0·3
Unknown solution	—	—	x
Water	2·4	2·1	$2{\cdot}1 - x$

After thorough mixing, the optical densities at 340 mμ. of cells 2 and 3 are measured, using cell 1 as reference. These readings are multiplied by the factor 2·7/3·0 to give D_2 and D_3 respectively. Immediately (zero time), 0·3 ml. of rabbit-muscle fraction *A* is added to each cell, the solutions are well

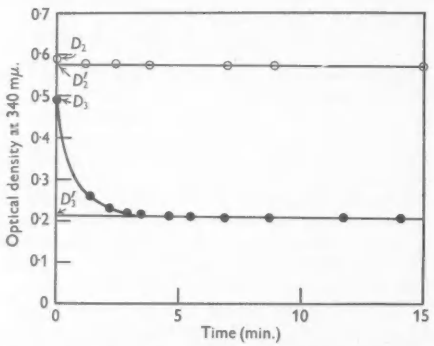

Fig. 1. Determination of hexosediphosphate by procedure *A*. 0·1 has been subtracted from all optical densities in the lower curve for ease of presentation. For description, see text.

mixed and readings taken against time for 10–20 min. Since the muscle fraction absorbs at 340 mμ., due to the presence of haematin compounds, the 0·3 ml. should be accurately pipetted into each cell. A typical measurement is shown in Fig. 1. It can be seen that cell 2 showed an immediate slight drop, followed by a very slow uniform decrease of the optical density. The immediate sudden drop is due to traces of substrates in the enzyme or reduced DPN preparations and is a blank to be applied to subsequent measurements. The slow uniform decline is probably due to autoxidizable flavoprotein in the enzyme preparation; in some preparations it is hardly detectable. The blank reading (D_2') is obtained by the extrapolation shown, which can be done very accurately. Cell 3 showed a fall of optical density at a decreasing rate until, after 4 min., the slow uniform decline shown by cell 2 was obtained. D_3' is obtained by extrapolation. The amount of HDP in the sample taken is given by $0{\cdot}241\ \{(D_3 - D_3') - (D_2 - D_2')\}$. The factor 0·241 was calculated from the extinction coefficient of reduced DPN (Horecker & Kornberg, 1948) and reaction (*A*). The blank reading need be determined only once each day and, with a four-compartment cell holder, it is possible to determine three unknown solutions simultaneously. The volume of unknown solution used should preferably contain between 0·03 and 0·08 μmole HDP. Tri(hydroxymethyl)aminomethane may be used as buffer in place of glycylglycine.

Procedure B (*a*). This differs from procedure *A* in the following respects. (i) In addition to the substances previously mentioned, the cells contain $MgCl_2$ (7 μmoles), ATP (0·25 μmole) and sufficient water to make the final volume 2·6 ml. D_2 and D_3 are obtained by multiplying the initial readings by 2·6/3·0. (ii) Immediately before adding the rabbit-muscle fraction *A*, 0·1 ml. of fraction *B* is added to each cell. Otherwise, the procedure and calculations are the same as above. Time is saved by preparing a stock solution containing the glycylglycine, $MgCl_2$, reduced DPN and ATP, which can be stored at −15° for 1–2 weeks.

Procedure *B* (*a*) estimates the HDP + HMP content. The HMP content is given by the difference between the values obtained by procedures *B* (*a*) and *A*.

Procedure B (*b*). This is the same as procedure *B* (*a*), except that hexosemonophosphate (synthetic glucose-6-phosphate is very suitable, since it does not contain any HDP) replaces ATP. The blank ($D_2 - D_2'$) is often somewhat greater in this measurement, since many samples of DPN contain some ~P. Procedure *B* (*b*) measures the HDP + ~P content. The ~P content is given by the difference between the values obtained by procedures *B* (*b*) and *A*.

Procedure C. To the solution to be analysed are added 0·3 ml. 0·5 M-phosphate buffer, pH 7·3; 0·1 ml. 0·25 M-glucose; 0·1 ml. M-NaF and water to make the final volume 2·8 ml. Hexokinase (0·1 ml.) and 0·1 ml. 0·15 M-$MgCl_2$ are added and, when the reaction is completed, 0·5 ml. 40% (w/v) trichloroacetic acid is added and the mixture centrifuged. The time required depends upon the activity of the hexokinase and should be determined in a separate experiment. An example with very dilute hexokinase is given in Table 1. If too long a period is allowed to elapse after complete reaction the final value may be slightly low due to the presence of a trace of phosphatase in some hexokinase preparations. A known volume (usually 2 ml.) of the supernatant is neutralized with N-KOH. A suitable sample of this solution is then analysed by procedure *B* (*a*). After multiplication by the various dilution factors, this gives the amount of ATP in the original solution. The amount of ADP equals the ~P, determined above, minus twice the ATP content.

For the complete analysis of a mixture of HDP, HMP, ATP and ADP as described above, rabbit-muscle fraction *A*

must contain very little phosphohexokinase. If phosphohexokinase is present, procedure *A* will give not HDP but HDP + HMP or HDP + ~P, whichever is the lower. Some preparations of rabbit-muscle fraction *A* contain sufficient phosphohexokinase to interfere and these preparations are unsuitable if both HDP and ~P or HMP are present. Dr R. K. Morton (private communication) has found that filtering fraction *A* through a pad of Super-Cel removed the phosphohexokinase almost completely.

Table 1. *Determination of ATP*

(0·1 ml. of stock solution of ATP treated by procedure *C* for different periods; 0·01 ml. crude hexokinase (0·5 mg. protein) used.)

Time (min.)	HMP found (μmoles)
2	1·02
4	1·38
10	1·49
20	1·50
60	1·54

EXAMINATION OF METHOD

Effect of glyceraldehydephosphate dehydrogenase in the enzyme mixture

If glyceraldehydephosphate dehydrogenase is present in the rabbit-muscle enzyme preparations, one might expect that part of the phosphoglyceraldehyde would reduce the DPN and thereby seriously interfere with the method described.

(7) Glyceraldehydephosphate + DPN + H_3PO_4 → diphosphoglyceric acid + reduced DPN (*glyceraldehydephosphate dehydrogenase*).

To test whether such interference was occurring under the conditions of the procedure described,

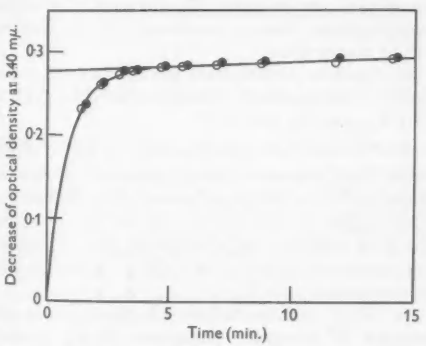

Fig. 2. Effect of iodoacetate on estimation of hexosemonophosphate by procedure *B* (*a*). ○, usual reaction mixture; ●, 0·001 M-iodoacetate added.

iodoacetate (0·001 M) was added to inhibit the glyceraldehydephosphate dehydrogenase. Cori, Slein & Cori (1948) have demonstrated that, under comparable conditions, 4×10^{-4} M-iodoacetate was sufficient completely to inhibit reaction (7). Fig. 2 shows that 0·001 M-iodoacetate caused no difference in the rate of oxidation of reduced DPN or in the amount oxidized. It follows that reaction (7) must be proceeding at an insignificant rate compared with reaction (3), a conclusion which is supported by the fact that the addition of inorganic phosphate or arsenate, which would be expected to increase the rate of reduction of DPN by glyceraldehydephosphate, did not affect the overall reactions studied. Reaction (3) is probably much faster than reaction (7), even in the presence of glyceraldehydephosphate dehydrogenase (Racker, 1947) and most of the latter enzyme remains in the supernatant after precipitation of rabbit-muscle fraction *A*.

Interfering substances

These are of two types: (*a*) those which oxidize reduced DPN rapidly in the presence of the muscle enzymes and are therefore erroneously included with the substance being analysed, and (*b*) those which react slowly with reduced DPN and therefore increase the rate of the slow decline after the oxidation of reduced DPN by the dihydroxyacetonephosphate is complete.

(*a*) The only substances of the first type which interfere with the estimation of HDP or HMP are pyruvate (or substances yielding pyruvate, namely phosphopyruvate and phosphoglycerate, see below) and oxaloacetate. The rabbit-muscle enzymes contain lactic and malic dehydrogenases which catalyse reactions (8) and (9) respectively

(8) Pyruvate + reduced DPN → lactate + DPN (*lactic dehydrogenase*)

(9) Oxaloacetate + reduced DPN → malate + DPN (*malic dehydrogenase*).

Pyruvate is readily determined by means of purified lactic dehydrogenase. Fig. 3 shows the separate determination of pyruvate and HDP in a mixture by the successive addition of lactic dehydrogenase and rabbit-muscle fraction *A*. Oxaloacetate may be determined by malic dehydrogenase (Straub, 1942) or after decarboxylation to pyruvate. The lactic dehydrogenase preparation described under Experimental contained sufficient malic dehydrogenase so that, in a suitable dilution, pyruvate and oxaloacetate could be separately determined, the pyruvate oxidizing the reduced DPN rapidly and oxaloacetate more slowly. Care is necessary if triosephosphates are present in the solution to be analysed, since some preparations of lactic dehydrogenase contain the enzymes necessary for reactions (2) and (3) in small concentrations, although quite free of aldolase.

Pyruvate and oxaloacetate also interfere with the determination of ~P. Another substance which also interferes with this measurement is creatine-

phosphate, since creatinephosphokinase, the enzyme catalysing reaction (10), is present in the preparations.

(10) Creatinephosphate + ADP → creatine + ATP (*creatinephosphokinase*).

70 % of creatinephosphate reacted in this way in 21 min., in one measurement. If creatinephosphate is present additional creatinephosphokinase should be added, so that reaction (10) proceeds to completion before the ADP is all used up by reaction (6) followed by reaction (5). The ~P analysis will then

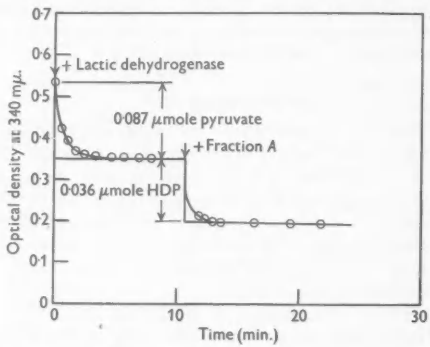

Fig. 3. The determination of pyruvate and hexosediphosphate on the same solution by the successive addition of lactic dehydrogenase and rabbit-muscle fraction *A*. Experimentally determined optical densities have been corrected for the dilution caused by the various additions.

include creatinephosphate as well as the reactive groups of ATP and ADP. The addition of purified creatinephosphokinase + 0·01 μmole of ADP has, in fact, proved to be a satisfactory method of estimating creatinephosphate (Morton, 1952; Kratzing & Narayanaswami, 1953). An alternative method of estimating creatinephosphate is to add creatinephosphokinase and ADP to the components of procedure *C*. In this case, however, careful timing of the reaction is necessary owing to the presence of a little phosphatase in the creatinephosphokinase preparation. Since the hexokinase preparations are free from creatinephosphokinase, creatinephosphate does not interfere with the estimation of ATP.

Phosphopyruvate, which is also an energy-rich phosphate compound, and phosphoglycerate, which can be considered as a potentially energy-rich phosphate compound, are also estimated as ~P, since the enzymes catalysing reactions (11) and (12) are present in the rabbit-muscle preparations.

(11) 3-Phosphoglycerate ⇌ 2-phosphoglycerate ⇌ phosphopyruvate (*phosphoglyceromutase and enolase*),

(12) Phosphopyruvate + ADP → ATP + pyruvate (*pyruvic phosphokinase*).

Since the pyruvate formed by reaction (12) will oxidize one molecule of reduced DPN (see above), each molecule of phosphoglycerate or phosphopyruvate will oxidize three molecules of reduced DPN. Thus the total ~P value will be somewhat overestimated if these substances are present in appreciable amounts. This is, however, usually unlikely. The method is a very sensitive procedure for the estimation of phosphoglycerate and phosphopyruvate, in the absence of other ~P compounds. Thus a solution of phosphopyruvate containing 2·02 μmoles total P/ml. gave on analysis 1·91 μmoles/ml. by the enzymic method assuming that 1 mol. phosphopyruvate oxidized 3 mol. reduced DPN; similarly, a solution of phosphoglyceric acid containing 8·4 μmoles total P/ml. gave 7·9 μmoles/ml. by the enzymic method. 1:3-Diphosphoglyceric acid has not been tested, but probably both phosphate groups will be estimated as ~P. Phosphopyruvate and phosphoglycerate will interfere in the same way as pyruvate in the estimation of HDP or HMP, since they are rapidly dephosphorylated in the presence of the rabbit enzymes, even in the absence of ADP. Possibly adenylic acid, present as an impurity in the DPN, is phosphorylated (maybe indirectly) with sufficient speed, although this reaction is very much slower than the phosphorylation of ADP.

(*b*) Substances which react slowly with reduced DPN include:

(i) α-Ketoglutarate (reaction 13)

(13) α-Ketoglutarate + NH_3 + reduced DPN → glutamate + DPN (*glutamic dehydrogenase*).

NH_3 is supplied by the $(NH_4)_2SO_4$ introduced with the enzyme preparations. The amount of glutamic dehydrogenase varies considerably from preparation to preparation.

(ii) Fructose-1-phosphate (procedures *B* (*a*) and *C* only) which is slowly phosphorylated by ATP to HDP by reaction (14).

(14) Fructose-1-phosphate + ATP → HDP + ADP.

(iii) High concentrations of glucose and fructose. These may react because of traces of hexokinase and fructokinase in the rabbit-muscle preparations. Provided sufficient phosphohexokinase is present, hexokinase and fructokinase will not interfere with the estimation of ~P.

(iv) High concentrations of glycogen in the presence of inorganic phosphate slowly liberate glucose-1-phosphate by the action of phosphorylase.

Substances which react slowly with reduced DPN do not affect the value of HDP, HMP or ~P obtained by the extrapolation; they only affect the slope of the line which is extrapolated. This is shown in the case of α-ketoglutarate in Fig. 4; 0·0495 μmole of HDP was found in the absence of α-ketoglutarate, while 0·0475 μmole was found in the presence of

5 μmoles α-ketoglutarate. The effect of fructose-1-phosphate is shown in Fig. 5. In this experiment, considerably more enzyme was added than usual (hence the high blank value). Nevertheless, the rate of oxidation of reduced DPN by as much as 0·6 μmole fructose-1-phosphate is not so great as to

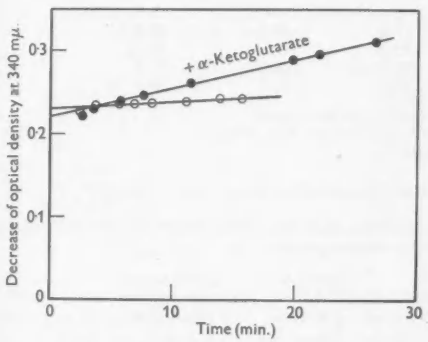

Fig. 4. Effect of α-ketoglutarate (5 μmoles) on the determination of hexosemonophosphate.

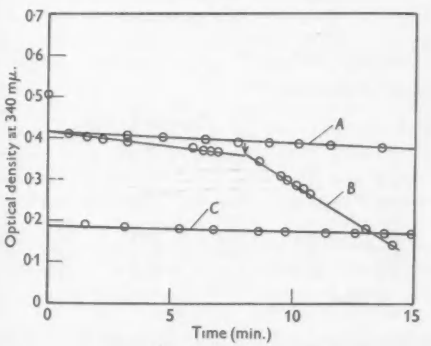

Fig. 5. Effect of fructose-1-phosphate. Curve *A* shows a blank determination without any added fructose-1-phosphate. Curve *B* was obtained when 0·1 μmole fructose-1-phosphate was initially present and a further 0·5 μmole was added at the arrow. The experimentally determined values of optical densities have been corrected for the dilution caused by this addition. Curve *C* shows the effect of 0·055 μmole glucose-6-phosphate without fructose-1-phosphate.

make the extrapolation impossible. Under the same conditions, all the fructose-6-phosphate was reduced in less than 2 min. It is clear that fructose-6-phosphate reacts very much more rapidly than fructose-1-phosphate in the presence of the rabbit-muscle fractions. Slein, Cori & Cori (1950) have shown that different enzymes are involved in the phosphorylation of these two compounds by muscle.

High concentrations of trichloroacetate considerably inhibit the enzymes and if the amounts of phosphorylated sugar or ~P are so small that a large sample of the acid extract must be used for analysis, it is preferable to deproteinize with perchloric acid, neutralize with 4N-KOH, cool to 0° and remove the potassium perchlorate by filtration (I am indebted to Dr C. C. Kratzing for this suggestion).

The following substances have been found to have no effect on the measurements—inorganic phosphate (unless the concentration is sufficient to precipitate magnesium), arsenate, malonate, fluoride, muscle adenylic acid, succinate, acetate, ethanol, TPN, DPN, ascorbic acid, inorganic pyrophosphate, ribose-5-phosphate. A large amount of cyanide (10 μmoles) decreased the value by about 10%. The lack of effect with fluoride shows that phosphatases are not causing any losses.

Reproducibility of the method

The reproducibility of the method for estimating HDP or HMP is excellent. The molarity of a solution of HDP, stored in the frozen state, was found to be 0·0352, 0·0340, 0·0336, 0·0360 and 0·0348 in successive analyses on different days, spread over a period of 3 months. The molarity of a solution of fructose-6-phosphate was similarly found to be 0·0338, 0·0331, 0·0326, 0·0332, 0·0331 in successive analyses.

Analyses of stock solutions of phosphorylated sugars

In Table 2 are reported analyses of stock solutions of various phosphorylated sugars, both by the present method and by determination of organic P. The latter was determined in two ways, (*a*) by acid digestion, and (*b*) by hydrolysis with purified phosphomonoesterase. All three methods gave excellent agreement in the case of enzymically prepared hexosemonophosphate (probably the equilibrium mixture of glucose-6-phosphate and fructose 6-phosphate) and fructose-1:6-diphosphate. In the case of glucose-1-phosphate, which was not determined by the phosphomonoesterase method, agreement between acid digestion and the enzymic method was also very close. The two other samples showed some disagreements which were further examined.

Both methods of estimating the organic P content of the solution of fructose-6-phosphate agreed and this was also in reasonable agreement with fructose-6-phosphate determined colorimetrically. The enzymic procedure described in this paper, however, gave values approximately 17% lower. The most likely explanation of this discrepancy is the presence of fructose-1-phosphate in the sample of fructose-6-phosphate, which was prepared commercially,

Table 2. *Analyses of stock solutions of phosphorylated sugars*

(Methods of preparation of stock solutions and analytical methods are described in the Experimental section. All values are given as μmoles/ml.)

Compound	Total P – inorganic P: Acid digestion	Total P – inorganic P: Phosphomonoesterase	Phosphorylated sugar measured by enzymic method
Glucose-1-phosphate*	9·8	—	9·6
Hexosemonophosphate (enzymic)	17·0	16·5	16·9
Fructose-6-phosphate†	40·5	40·1	33·2‡
Glucose-6-phosphate	46·0	41·0	37·3
Fructose-1:6-diphosphate	21·2	20·7	19·9

* 9·9 μmoles/ml. calculated from weight of crystals.
† 41·9 μmoles/ml. measured colorimetrically (Roe, 1934).
‡ Including 0·28 μmole/ml. HDP.

Table 3. *Partial hydrolysis of synthetic glucose-6-phosphate by phosphomonoesterase*

(A very dilute preparation of highly purified phosphomonoesterase was used; the erratic course of the hydrolysis/time curve is probably due to different degrees of surface inactivation in different tubes.)

Time of hydrolysis (hr.)	Inorganic P (μatoms)	ΔP (μatoms)	Glucose-6-phosphate (μmoles)	Δ Glucose-6-phosphate (μmoles)
0	0·01	—	1·86	—
0·17	0·01	0	1·88	0·02
0·5	0·55	0·54	1·23	−0·63
1	0·60	0·59	1·25	−0·61
3	1·04	1·03	0·83	−1·03
36	2·01	2·00	0·04	−1·82

Table 4. *Analyses of solutions of ATP and ADP*

(All concentrations are expressed as μmoles/ml.)

	Commercial ATP	Laboratory ATP				Laboratory ADP		
Inorganic P	11·6	1·7	1·6	1·7	—	0·8	—	—
ATP	6·6	12·4	23·9	19·3	21·7	0	0	—
~P	19·9	25·1	48·5	39·0	48·9	6·8	19·1	20·9
Acid-labile P	25·7	28·5	51·4	42·4	—	7·4	—	—
ADP*	6·7	0·3	0·7	0·4	5·5	6·8	19·1	20·9
ATP + ADP	13·3	12·7	24·6	19·7	27·2	6·8	19·1	20·9
Total adenosine	12·9	14·2	25·4	19·8	26·9	6·6	19·0	21·4

* Calculated (~P − 2 ATP).

probably by acid hydrolysis of fructose-1:6-diphosphate. An acid hydrolysis curve revealed the existence of a small amount of a more rapidly hydrolysable component, which is in agreement with this hypothesis. Fructose-1-phosphate would be indistinguishable from fructose-6-phosphate by all the methods used in Table 2, except the enzymic procedure.

The HMP content of the sample of synthetic glucose-6-phosphate was only 81 % of that expected from the organic P content. The phosphate liberated by the phosphomonoesterase was, however, considerably less than the total organic P. Table 3 shows close agreement between HMP disappearing and inorganic P appearing after partial hydrolysis of the preparation. It appears that the glucose-6-phosphate preparation contains an organic phosphorus compound which does not react with the rabbit-muscle enzymes and is only partially hydrolysable by phosphomonoesterase. Ochoa *et al.* (1950) found that only 80 % of a sample of synthetic glucose-6-phosphate (presumably standardized by total P determination) reduced TPN in the presence of glucose-6-phosphate dehydrogenase.

Analyses of solutions of ATP and ADP

Table 4 shows analyses of solutions prepared from samples of ATP obtained commercially and of ATP and ADP prepared in the laboratory. In all samples, except one, the ATP + ADP agreed closely with the total adenosine content calculated from the absorption at 260 mμ. Most laboratory-made ATP preparations contained practically no ADP (in the case of the exception, treatment with trichloroacetic acid was unduly delayed after the death of the animal). The commercial sample of ATP contained

as much ADP as ATP, as well as large amounts of inorganic P. The acid-labile P in all cases exceeded the ~P determined enzymically, the discrepancy being considerable only in the case of the commercial ATP. The discrepancy is probably due to pyrophosphate (Bailey, 1949).

Table 5. *Analysis of ADP prepared by Bielschowsky's method*

	μmoles/ml.
Inorganic P	4·1
ATP	4·3
~P	18·1
ADP*	9·5
ATP + ADP	13·8
Total adenosine	20·3
Acid-labile P	18·1
Acid-labile P/total adenosine	0·90

* Calculated (~P − 2 ATP).

The method of Bielschowsky (1950) for preparing ADP (hydrolysis at pH 4·5, in the presence of Mg^{++}) was followed, using the commercial sample of ATP, which already contained as much ADP as ATP. The analysis of the final product is given in Table 5. Although the ratio of acid-labile P to total adenosine is close to the theoretical for ADP (1·0), the product contains considerable ATP and much material absorbing at 260 mμ. not accountable in terms of ATP + ADP. This latter material is very likely adenylic acid. Thus, even though all the ATP is not hydrolysed, much of the ADP has been further hydrolysed to adenylic acid. These results give little reason to hope that ADP can be satisfactorily prepared from ATP by controlled acid hydrolysis. A weakness of the conventional chemical methods for following such reactions is that a mixture of equimolar proportions of ATP and adenylic acid gives the same result as pure ADP. The agreement between the ~P and acid-labile P in Table 5 suggests that all the pyrophosphate originally present in the commercial ATP has been removed.

DISCUSSION

The methods described in this paper have proved very useful for a number of investigations. In common with similar procedures developed by Ochoa and his associates, they combine the advantages of the specificity obtained by using enzymes with the sensitivity of spectrophotometric methods. Although a few compounds do interfere, none of these has actually been present in any of the problems investigated by these methods. The specificity of enzymic methods gives them the very great advantage over chemical methods that physical separations of different phosphorus compounds are not necessary. They are particularly valuable for the estimation of small amounts of phosphorylated sugars or adenine nucleotides in the presence of large amounts of inorganic phosphate. Inorganic phosphate must be removed before application of chemical methods to organic phosphorus compounds, and when there is a large excess of inorganic phosphate it cannot be precipitated without large losses of organic phosphate by co-precipitation (Lehninger, 1949; Ennor & Rosenberg, 1952).

The methods described in the present paper determine in a mixture (*a*) total hexosemonophosphate, (*b*) hexosediphosphate + triosephosphates, (*c*) ATP and (*d*) ~P. No attempt has been made to measure the individual hexosemonophosphates separately or to distinguish hexosediphosphate from triosephosphate. In oxidative phosphorylation experiments, for example, the esterified phosphate is often transferred to glucose by means of hexokinase. Since yeast hexokinase preparations contain the active hexosemonophosphate isomerase, the esterified phosphate will appear as both glucose-6-phosphate and fructose-6-phosphate and the total hexosemonophosphate found will correctly measure the esterification of inorganic phosphate. If separate determinations are required, this could probably be achieved either by purification of the rabbit-muscle enzymes or by physical separation of the esters, for example by paper chromatography (Hanes & Isherwood, 1949).

The method described is not the only enzymic method available for the estimation of hexosemonophosphate. Ochoa *et al.* (1950), Slein (1950) and Kornberg & Pricer (1951*b*) have used the reduction of TPN by glucose-6-phosphate, catalysed by glucose-6-phosphate dehydroganase. Slein (1950) determined glucose-6-phosphate + fructose-6-phosphate by addition of hexosephosphate isomerase. By the further addition of phosphomannose isomerase the method becomes specific for mannose-6-phosphate. Mannose-6-phosphate has not been tested in the present work, but since Slein (1950) found that phosphomannose isomerase was precipitated from rabbit-muscle extract between 0·45 and 0·55 saturation with ammonium sulphate, it is very likely that this enzyme is present in the rabbit-muscle fractions used in the present paper and that mannose-6-phosphate, if present, would be estimated with the other hexosemonophosphates. Kornberg & Pricer (1951*b*) have recently adapted the method using glucose-6-phosphate dehydrogenase to the determination of ATP and ADP by the separate addition of hexokinase and myokinase. No special advantages of the present procedure over the methods used by the above authors is claimed. Which method is used will be determined by convenience and the interfering substances likely to be present. Since, in the present method, each molecule

of hexosemonophosphate reacts with two molecules of reduced DPN whereas only one molecule of TPN is reduced by glucose-6-phosphate, the former method has double the sensitivity. This will not often be an advantage, because both methods are highly sensitive, but was an important consideration in choosing the present method for studying phosphorylation coupled with the reduction of cytochrome *c* (Slater, 1950).

Vishniac & Ochoa (1952) have recently determined HDP by the reduction of DPN catalysed by purified aldolase, triosephosphate isomerase, glyceraldehydephosphate dehydrogenase, ADP and the enzyme which catalyses the phosphorylation of ADP by diphosphoglyceric acid. Special care must be taken to remove glycerolphosphate dehydrogenase from the enzymes if this method is used (Racker, 1947) and it is not as convenient for most purposes as the method described in this paper. Vishniac & Ochoa's (1952) special problem was to estimate small quantities of HDP in the presence of large amounts of 3-phosphoglyceric acid, which interferes with the present method.

Kornberg & Pricer (1951*b*) have also introduced a more specific method for the estimation of ADP by the use of phosphoenol pyruvic acid and pyruvic phosphokinase in the presence of lactic dehydrogenase and reduced DPN. According to these authors, ADP is the specific acceptor and, for every molecule present, one molecule of pyruvate appears and oxidizes the equivalent amount of reduced DPN.

The sensitivity of the enzymic methods is such as to lend them to the estimation of adenine nucleotides in very small amounts of biological materials. The optimal amount of ATP to be taken for the estimation is only 0·025 μmole in 3 ml., and 0·01 μmole can be determined with considerable accuracy. If microcells were used, this figure could be greatly decreased.

SUMMARY

1. An enzymic method for the determination of phosphorylated sugars and energy-rich compounds is described. The method depends upon the enzymic conversion of these compounds to dihydroxyacetonephosphate, which then reacts with reduced DPN in the presence of glycerolphosphate dehydrogenase. The amount of reduced DPN reacting is determined spectrophotometrically.

2. The method is highly sensitive, 0·05 μmole of phosphorylated sugar or energy-rich phosphate being measured with an accuracy of a few per cent.

3. In a complex mixture separate analyses are obtained for (*a*) hexosediphosphate + triosephosphates, (*b*) hexosemonophosphates (glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, but not fructose-1-phosphate), (*c*) ATP and (*d*) other energy-rich compounds (ADP, creatine-phosphate, phosphopyruvate (which is over-estimated by 50%)).

4. The only substances which interfere are (i) pyruvate and oxaloacetate, each molecule of which reacts as one-half a molecule of hexosediphosphate and (ii) phosphoglycerate, which behaves like phosphopyruvate. Pyruvate and oxaloacetate may be separately determined with lactic and malic dehydrogenases.

5. Analyses of preparations of phosphorylated sugars and adenine nucleotides, either prepared in the laboratory or obtained commercially, have been made both by the new method and by conventional chemical methods. Agreement was very close in most cases. Where there was disagreement this has been traced to the presence in the preparations of impurities which are estimated by the chemical but not by the enzymic method.

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The Effect of Thiol and other Group-specific Reagents on Erythrocyte and Plasma Cholinesterases

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That the activity of certain enzymes may depend on the presence in the enzyme molecule of intact sulphydryl groups is now widely accepted and the cholinesterases have been considered to belong to this class of 'sulphydryl enzymes' (Nachmansohn & Lederer, 1939; Barron & Singer, 1943; Stadie, Riggs & Haugaard, 1945; Thompson, 1948). Many of the relevant observations were, however, made before it was realized that cholinesterases from different sources are not identical in specificity and other properties (Alles & Hawes, 1940; Richter & Croft, 1942; Mendel & Rudney, 1943; Zeller & Bissegger, 1943), and a critical survey of the literature reveals that the evidence for regarding any one cholinesterase as an —SH enzyme rests almost entirely upon inhibition by a few reagents, some by no means specific for —SH groups. The present work was undertaken with the object of investigating the possible sulphydryl nature of the cholinesterases of the human plasma and erythrocytes and attempting to decide whether sulphydryl groups can be regarded as having functional significance in relation to the activity of these representative mammalian cholinesterases.

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Table 1 summarizes existing work with —SH inhibitors. The most extensive observations are those of Nachmansohn & Lederer (1939) who found that the cholinesterase of the electric organ of the torpedo was inhibited by such typical thiol reagents as copper, maleic acid, iodoacetate, oxidized glutathione and alloxan. They concluded that torpedo cholinesterase was an —SH enzyme, though with some of these reagents rather large concentrations and prolonged incubation periods were required to produce a significant inhibition. Moreover, the specificity of some of these reagents is doubtful, while other thiol reagents, such as arsenite, were not tested. Mapharside (3-amino-4-hydroxyphenylarsenoxide) was, however, found by Barron & Singer (1943) to be fairly powerful as an inhibitor of a cholinesterase of unspecified origin (electric-organ cholinesterase) while Thompson (1947) found that pigeon-brain cholinesterase was fairly sensitive to arsenite. Thompson (1948) also reported that pigeon-brain cholinesterase, unlike pigeon-brain 'pyruvate oxidase', could be protected from arsenite by a monothiol, cysteine ester hydrochloride, as well as by the dithiol 2:3-dimercaptopropanol (BAL). He tentatively suggested that cholinesterase is a monothiol enzyme, that is, its activity possibly depends on the presence of one thiol group per active centre in contrast to the 'dithiol enzyme' of pyruvate oxidation which is presumed to form an arsenic complex of stability intermediate between those which arsenic forms with cysteine and with BAL.

Horse serum cholinesterase was found by Massart & Dufait (1939) to be inhibited by arsenite in fairly high concentration. Mackworth (1948) found that this enzyme is not markedly sensitive, as are succinic and triosephosphate dehydrogenases, activated papain and other typical —SH

Table 1. *The effect of thiol reagents on cholinesterases, a résumé of earlier work*

Reagent	Enzyme source	pI*	Inhibition (%)	Author
Arsenite	Horse serum	1·7	87	Massart & Dufait (1939)
	Pigeon brain	4	52	Thompson (1947)
Lewisite	Horse serum	2·4	30	Mackworth (1941)
	Serum†	4	85	Barron *et al.* (1947)
	Pigeon brain	3	19	Thompson (1947)
Phenyldichloroarsine	Pigeon brain	3	57	Thompson (1947)
3-Amino-4-hydroxyphenylarsenoxide	Electric organ (?)	4·2	57	Barron & Singer (1943)
Copper	Torpedo	3·3	38	Nachmansohn & Lederer (1939)
	Horse serum	2·7	87	Mackworth (1941)
Maleic acid‡	Torpedo	1·5	22	Nachmansohn & Lederer (1939)
Iodoacetate‡	Torpedo	1·7	88	Nachmansohn & Lederer (1939)
Iodoacetamide	Cat serum	?	Nil	Wels & Repke (1947)
Glutathione‡ (oxidized)	Torpedo	2	24	Nachmansohn & Lederer (1939)
Iodine‡	Torpedo	5·75	85	Nachmansohn & Lederer (1939)
Alloxan§	Torpedo	3·4	38	Nachmansohn & Lederer (1939)
o-Iodosobenzene	Human plasma	?	Nil	Stadie *et al.* (1945)
Bromobenzyl cyanide	Horse serum	1·6	70	Mackworth (1948)
Chloropicrin	Horse serum	1·6	Nil	Mackworth (1948)
Bromoacetophenone	Horse serum	3	84	Mackworth (1948)
Ethyl iodoacetate	Horse serum	2·8	Nil	Mackworth (1948)
Oxygen (high pressure)	Rat brain	—	Nil	Stadie *et al.* (1945)

* Calculated from authors' data where necessary.
† Species not stated.
‡ Partly reactivated by glutathione.
§ Incubated with enzyme 4–10 hr.

enzymes, to lachrymators which are known (Bacq, 1946; Dixon, 1948) to combine readily with protein —SH groups. Typical —SH inhibitors to which various cholinesterases appear to be rather insensitive are lewisite (Mackworth, 1941; Thompson, 1947; but contrast Barron, Miller, Bartlett, Meyer & Singer, 1947), phenyldichloroarsine (Thompson, 1947), iodoacetamide (Wels & Repke, 1947) and *o*-iodosobenzene (Stadie *et al.* 1945) which is generally inhibitory to —SH enzymes (Dickens, 1946). Finally, the difference in sensitivity of cholinesterases of different origin to certain inhibitors listed in Table 1 underlines the need for comparative studies.

MATERIALS AND METHODS

Enzyme preparations

Human erythrocyte cholinesterase. A preparation obtained by the method of Mounter & Whittaker (1950) was further purified by precipitation with $(NH_4)_2SO_4$ and differential centrifugation of the dispersed and dialysed precipitate (for details see Mounter, 1952). It gave 5700 μl. acid (or CO_2)/mg. N/hr. using 30 mM-acetyl-β-methylcholine as substrate.

Human plasma cholinesterase. This was prepared from reconstituted dried transfusion plasma by the method of Strelitz (1944) (stage 3 of her preparation). Initial activity 150 μl./mg. dry wt./hr.

Reagents and substrates

Acetyl-β-methylcholine chloride (30 mM) and, in a few experiments, acetylcholine perchlorate (6 mM), were used as substrates for erythrocyte cholinesterase; acetylcholine perchlorate (15 mM) was the substrate for plasma cholinesterase. The reagents employed for inhibition studies were the best commercial grade, or laboratory samples available from previous work. Acid and alkaline reagents were brought to pH 7·4 before use. Lewisite and diphenylchloroarsine were dissolved in a small volume of *iso*propanol and diluted with 0·2% $NaHCO_3$ before use. The amount of alcohol thus added to the enzyme was found in control experiments to be without effect. Oxidized glutathione was kindly provided by Dr R. V. Cecil.

Methods

Determination of enzyme activity was made by Ammon's (1933) modification of the Warburg technique, in which CO_2 displaced from a $NaHCO_3$-CO_2 buffer at pH 7·4 and 38° by acid produced during hydrolysis is measured manometrically. The solution in each Warburg flask (total vol. 3 ml.) contained 0·2% $NaHCO_3$ in equilibrium with 5% CO_2 in N_2 as the gas phase. The enzyme and reagent were contained in the main compartment of the flasks and the substrate in the side bulb; in reactivation experiments, the reactivating agent was placed in the second side bulb of double side-bulb flasks. The flasks were attached to the manometers, gassed for 3 min. and placed in the thermostat. After equilibrating for 10 min. the substrate was added from the side bulb and readings commenced 5 min. later. In reactivation experiments the initial incubation was continued for 15 min., the reactivator was then added and the incubation continued for a further 10 min. before addition of the substrate. The inhibition due to reactivator was controlled separately. Rates of hydrolysis and percentage inhibitions were constant during the experimental period. Enzyme and inhibitor were usually in contact 20 min. before addition of substrate and in some experiments with arsenite and mercurials in which the time of contact was varied, this was found to be adequate for the attainment of maximum inhibition.

In a few experiments the photometric method of Hestrin (1949) was used. All experiments included controls without enzymes.

RESULTS

Inhibition experiments with thiol reagents

Results obtained at pH 7·4 are summarized in Table 2. Where possible, the pI_{50} value (pI_x denotes the Briggsian logarithm of the molar concentration of inhibitor required to produce x% inhibition, with sign reversed) has been obtained by interpolation. When 50% inhibition was not attained, the pI_{50} value is recorded as 'less than' the highest pI value used, and the inhibition corresponding to this pI value is given in an adjoining column.

Table 2. *Inhibition of cholinesterases by thiol reagents*

	Red-cell cholinesterase		Plasma cholinesterase	
Reagent	pI_{50}	Inhibition (%) at highest pI used	pI_{50}	Inhibition (%) at highest pI used
Sodium arsenite	4·2	—	3·4	—
Lewisite	<3	22	<3	12
Phenylarsenoxide	<3	35	<3	25
Diphenylarsine chloride	<3	31	<3	26
Phenylmercuric nitrate	<3	15	4	—
p-Chloromercuribenzoic acid	2·2	—	2·6	—
Mercuric chloride	2·6	—	3·8	—
Copper sulphate	<2	45	3·7	—
Silver nitrate	<3	0	<3	10
Potassium antimonyl tartrate	<2	10	<2	10
Stibacetin	<3	5	<3	0
Sodium maleate	<1	20	<1	12
Sodium iodoacetate	<2	10	<2	5
Glutathione (oxidized)	<2	0	<2	0
Potassium ferricyanide	<2	0	<2	0
Iodine	3·6	—	3·6	—

Arsenicals. Appreciable inhibition was obtained with sodium arsenite, the erythrocyte enzyme being more sensitive than that of the plasma. In experiments with varying times of contact of enzyme and inhibitor before addition of substrate (5–60 min.) inhibition was found to be maximal at 5 min. By contrast, the inhibiting effect of organic arsenicals, normally very powerful inhibitors of sulphydryl enzymes (reviewed by Stocken & Thompson, 1949), is much less than the inorganic arsenite ion, as was noted for pigeon-brain and horse-serum cholinesterase by Thompson (1947) and Mackworth (1941).

As the As—S link is readily hydrolysed in alkaline solution (Cohen, King & Strangeways, 1931) it was thought to be of some importance to determine the effect of pH on the inhibition of blood cholinesterases by arsenite. In the experiments with erythrocyte cholinesterase, acetylcholine (2·2 mM) and the enzyme were incubated at 38° in 0·034 M-phosphate buffer of the required pH, in the presence and absence of 0·5 mM-arsenite. Other tubes, containing substrate, but no enzyme or inhibitor, or buffer alone, were run as a non-enzymic control and as a reagent blank respectively. Samples (1 ml.) were withdrawn at 20 min. intervals and the acetylcholine content determined by Hestrin's (1949) method. Experiments with plasma cholinesterase had 5·5 mM-acetylcholine and 1 mM-arsenite, but were otherwise identical. Rates of hydrolysis were linear with time, and from them the percentage inhibition was calculated in the usual way. Fig. 1 shows the inhibition plotted as a function of pH. The values obtained at pH 7·4 with the Warburg technique are inserted for comparison; the agreement is satisfactory considering that both buffer and substrate concentration were different. It will be seen that the inhibition increases with increasing pH and is maximal at the highest pH used (pH 8). If the arsenic had been combining with a monothiol grouping, one would have expected the inhibition to be maximal on the acid side of neutrality as an alkaline reaction favours dissociation of thioarsenites. This point is discussed more fully below.

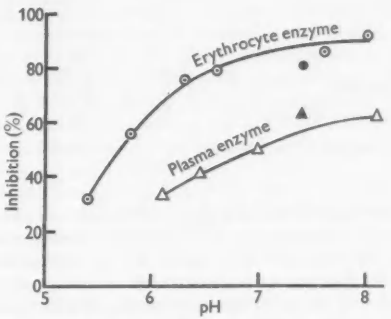


Fig. 1. Effect of pH on inhibition of cholinesterases by arsenite (0·5 mM, erythrocyte enzyme; 1·0 mM, plasma enzyme).

Mercurials. The readiness of thiol groups to combine with mercury compounds is one of their earliest recorded properties. In the present study, mercuric chloride, phenylmercuric nitrate, and *p*-chloromercuribenzoic acid were tried as inhibitors. Of these substances, mercuric chloride was the most powerful inhibitor of plasma cholinesterase, phenylmercuric nitrate was also effective, but *p*-chloromercuribenzoate only gave 22% inhibition at 1 mM. Erythrocyte cholinesterase is only slightly inhibited by all these substances. No increase in inhibition was found on prolonged incubation with the mercurials.

Other thiol reagents. Iodoacetate, oxidized glutathione, ferricyanide and maleate had little or no effect on either enzyme; incubation for as long as 4 hr. with maleate produced no more than 10% inhibition. Iodine strongly inhibited both cholinesterases.

Reversal of heavy-metal inhibition by thiols

The ability of mono- and di-thiols and of a 1:2-diol, catechol, to reverse the inhibitory action of heavy metals was also examined. The results are presented in Table 3. Taking first the results with plasma cholinesterase, it will be seen that the dithiol BAL is consistently effective in reversing the inhibition due to arsenic, mercury or copper. By contrast, the monothiols show variation in effectiveness. Thus 2-mercaptoethanol, but not glutathione or cysteine, was effective against arsenite, but all were fully effective against mercury and copper. Catechol was uniformly ineffective.

Experiments with the erythrocyte enzyme were limited by the very high concentration of most inhibitors required to produce appreciable inhibition. At the same concentrations relative to the inhibitor as were used with the plasma enzyme, BAL was

Table 3. *Reversal of metal inhibition of cholinesterases by thiols*

Inhibitor (*I*)	Concn. (mM)	Reactivator (*R*)	Concn. (mM)	Inhibition (%) (*I*)	Inhibition (%) (*I* + *R*)	Inhibition (%) (*R*)	Reactivation (%) $100\frac{I-(I+R)}{I-R}$
			A. Plasma cholinesterase				
Arsenite	1	BAL	10	56	0	0	100
	1	BAL	3	60	0	4	107
	0·3	2-Mercaptoethanol	10	49	20	4	65
	1	2-Mercaptoethanol	10	63	28	5	60
	0·3	Glutathione	10	48	42	2	13
	1	Glutathione	10	65	58	14	14
	0·3	Cysteine	10	44	44	20	0
	1	Cysteine	10	69	60	22	19
	1	Catechol	10	64	62	0	3
Mercuric chloride	0·5	BAL	5	97	11	5	94
	0·5	2-Mercaptoethanol	10	98	0	0	100
	0·5	Glutathione	10	98	25	6	80
	0·5	Cysteine	10	98	13	16	103
Phenylmercuric nitrate	1	BAL	10	100	18	10	91
	1	2-Mercaptoethanol	10	94	32	0	66
Copper sulphate	0·3	BAL	5	62	12	10	96
	0·3	2-Mercaptoethanol	10	62	4	1	95
	0·5	Glutathione	10	80	20	15	92
	0·3	Cysteine	10	65	30	20	78
			B. Erythrocyte cholinesterase				
Arsenite	1	BAL	10	98	35	33	97
	1	BAL	3	95	30	29	99
	1	2-Mercaptoethanol	10	92	82	6	12
	0·5	2-Mercaptoethanol	10	85	59	6	33
	1	Glutathione	10	94	90	3	4
	0·3	Glutathione	10	85	82	4	4
	1	Cysteine	10	98	90	6	9
	0·3	Cysteine	10	83	90	18	None
	1	Catechol	10	91	92	7	None
Lewisite	1	BAL	10	25	33	30	Reactivator more toxic than inhibitor
Mercuric chloride	0·5	BAL	10	11	23	25	
Phenylmercuric nitrate	1	BAL	10	12	27	25	

actually more toxic to the enzyme than lewisite or mercury; it was, however, an effective antidote for arsenite, while glutathione, cysteine and catechol were not. 2-Mercaptoethanol proved less effective than in the experiments with the plasma enzyme.

Experiments with reagents other than thiol reagents

A few experiments were done with reagents specific for other groups. Table 4 shows that neither enzyme is sensitive to reagents for carbonyl ester groups or iron or to chelating agents. A possible significant degree of inhibition was obtained with 8-hydroxyquinoline.

esterases can inhibit unpurified preparations in extremely low concentrations.

The problem of interpreting conflicting results with thiol reagents has been discussed *inter alia* by Hellerman (1937) and by Barron & Singer (1945). The latter authors stress possible variations in the reactivity of 'essential' —SH groups and conclude that the arsenicals and *p*-chloromercuribenzoic acid are the most reactive —SH reagents. Our results show that neither cholinesterase is markedly sensitive to *p*-chloromercuribenzoic acid, though the plasma enzyme is fairly sensitive to other mercurials, and among the various arsenicals tested, only

Table 4. *Effect of reagents (other than thiol reagents) on blood cholinesterases*

Reagent	Diagnostic for	Concn. (mM)	pI	Inhibition (%) Erythrocyte	Inhibition (%) Plasma
Hydroxylamine	—CO— —COOR	5	2·3	0	0
Phenylhydrazine	—CO—	5	2·3	15	0
Semicarbazide	—CO—	5	2·3	0	0
Sodium bisulphite	—CO—	10	2	41	25
Sodium cyanide	Fe	1	3	0	0
Sodium azide	Fe	1	3	0	0
Thiourea	Metals	1	3	0	0
8-Hydroxyquinoline	Metals	3	2·5	20	67

DISCUSSION

The results presented above are inconclusive in that the functional groups of the two cholinesterases studied have not been identified. Only two inhibitors having pI_{50} values of 4 or more were encountered: sodium arsenite (rod-cell cholinesterase) and phenylmercuric nitrate (plasma cholinesterase). The plasma enzyme was moderately sensitive ($pI_{50} > 3$) to AsO_3^{3-}, Hg^{2+} (as chloride), Cu^{2+} (as sulphate) and both were fairly sensitive to iodine ($pI_{50} = 3{\cdot}5$). Both enzymes were usually inhibited by metallic —SH reagents in sufficiently high concentration, but clearly differed from typical —SH enzymes in being either much less sensitive or quite insensitive to most —SH reagents tested. The results are thus generally in line with those obtained with other cholinesterases (Table 1), and while certain differences in sensitivity undoubtedly exist, the resemblances between the cholinesterases are more striking than these differences. It is unlikely that the pattern of the results would have been different if more highly purified enzymes had been used as most of the work with typical —SH enzymes has been done with impure preparations, and the amount of the less inhibiting reagents was frequently greatly in excess of the total protein content of the reaction mixture. It seems inconceivable that inert protein could take up many times its weight of reagent before any thiol groups in the enzyme were attacked, especially as the specific anticholin-

sodium arsenite was an effective inhibitor, although this is usually less inhibitory than the organic arsenicals to arsenic-sensitive enzymes. A possibly significant comparison may be made between the cholinesterases and D-amino-acid oxidase, another enzyme whose status as an —SH enzyme is doubtful. Like the cholinesterases, this enzyme is sensitive to trivalent arsenic and mercurials (Barron & Singer, 1943) but not to lachrymators (Mackworth, 1948) or high pressure oxygen (Dickens, 1946). Conceivably, the cholinesterases and D-amino-acid oxidase have the same group in their active centres which may be an —SH group strongly modified by a neighbouring group, a group capable of being converted to an —SH group by some but not all —SH inhibitors, or some quite different group possessing some sulphydryl properties.

Goldstein & Doherty (1951) have reached the conclusion that the inhibition of plasma cholinesterase by mercuric chloride is not due to reaction with 'essential' thiol groups. Several of their observations are, however, in conflict with ours. They found that mercuric chloride inhibition was progressive, the time course of inhibition being dependent on mercuric chloride concentration, that a 2·5-fold excess of BAL was not able to effect reactivation of the enzyme after inhibition and that the enzyme was uninhibited by 3 mM-*p*-chloromercuribenzoic acid. In our experiments, inhibition by mercuric chloride was always complete in less than 30 min., a tenfold excess of BAL was able to

effect almost complete reactivation of the enzyme and 2·5 mM-*p*-chloromercuribenzoic acid gave 50% inhibition. Our preparation also appeared to be more sensitive to mercuric chloride than theirs.

Hydrogen-ion concentration is known to affect the reactivity of —SH groups and the stability of mercaptides. It is conceivable, though unlikely, that the relatively feeble inhibition of these enzymes by arsenicals might be due to dissociation of the

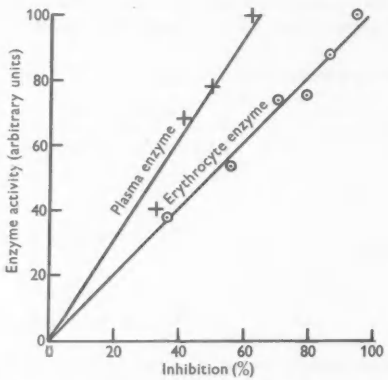
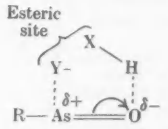


Fig. 2. Correlation between cholinesterase activity and inhibition by arsenite.

putative thioarsenite link of the arsenical-enzyme complex, at the slightly alkaline pH of the experiment. We have found, however, that arsenite inhibition actually decreases with decreasing pH, though this should favour thioarsenite formation. Indeed, there exists a good correlation between enzyme activity and the inhibition produced by a given concentration of arsenite, as is shown in Fig. 2. This suggests an alternative explanation of the inhibitory effect of arsenic. Wilson & Bergmann (1950*a*, *b*) have explained the effect of pH on the activity of electric-organ cholinesterase and inhibition by certain anticholinesterases by postulating the existence, in the part of the active centre concerned with fission of the ester link of the substrate, of a group, or groups, which (*a*) act as a base and accept a proton in acid solution, and (*b*) act as an acid and liberate a proton in alkaline solution. The forms produced by addition of a proton to the basic group (denoted by Y− in structure I) or removal of a proton from the acidic group (XH in I) are supposed to be enzymically inactive. The nucleophilic proton-accepting basic group is supposed to form a link with the electrophilic carbon of the acyl group of a substrate molecule, while the acidic group forms a link (possibly a hydrogen bond) with one or other of the ester oxygens, leading to the electronic shifts which disrupt the ester link. The co-operation of both these groups is conceived to be necessary for rupture of the link.

It seems certain that a large number of structures other than carboxylic esters would also interact, to varying extents, with such a system and would thereby possess varying degrees of anticholinesterase activity which would vary with the pH in a similar way to the enzyme activity. If this hypothesis can be generalized it may account for the anticholinesterase activity not only of L-amino-acids (Bergmann, Wilson & Nachmansohn, 1950), and of many organic phosphoric esters, but also of inorganic pyrophosphate, citrate, tartrate, atoxyl, and finally arsenite and related organic arsenicals (I) (for literature, see Augustinsson, 1948). The fact



(I)

that atoxyl is to some extent inhibitory is significant for the point of view expressed here as pentavalent arsenicals are normally non-inhibitory to —SH enzymes.

The only non-metallic —SH reagent appreciably inhibitory was iodine. Torpedo cholinesterase is also sensitive to this reagent (see Table 1). Iodine is however a highly reactive substance and oxidation of —SH groups is only one of many ways in which it could conceivably inactivate an enzyme.

Both cholinesterases were quite insensitive to group-specific reagents other than —SH reagents with the possible exception of hydroxyquinoline ($pI_{20} = 2{\cdot}5$, erythrocyte; $pI_{67} = 2{\cdot}5$, plasma). Since the other chelating agent tested, thiourea, was non-inhibitory, this can hardly be regarded as evidence for the participation of a metal ion in the active centre.

In conclusion, there seems to be little or no evidence for the participation of —SH groups, carbonyl groups, or metal ions in the active centres of these enzymes. No class of group-specific reagents is consistently inhibitory, and we believe that such inhibitions as have been observed with individual members of these classes are probably to be explained by a non-specific reaction of the substance concerned. In our present state of knowledge of the constitution of the active centres of these enzymes, it is hardly possible to decide just what these non-specific reactions are. Further studies of the effect of pH on them might be of value.

SUMMARY

1. The inhibitory effects of a number of thiol reagents and other group-specific reagents on the cholinesterases of human blood have been studied.

2. Although the enzymes were moderately sensitive to arsenite and iodine, and were usually inhibited by other heavy metal reagents in sufficiently high concentration, they differed from typical —SH enzymes in being either much less sensitive or quite insensitive to most —SH reagents tried. Further, arsenite inhibition was reduced by pH, not increased as would be expected if a thio-arsenite link had been formed.

3. It is concluded that these cholinesterases are in all probability not —SH enzymes and that there is also no evidence for the participation of metal ions, carbonyl groups or carboxylate groups in the active centre.

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The Effect of Dosage on Sexual Differences in the Iron Metabolism of Rats

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Both rats and human beings exhibit sexual differences in the metabolism of iron. Widdowson & McCance (1948) outlined the information available and showed that, given equal opportunities, female rats stored much more iron in their livers than males. They also showed that this was true of some but not of all other species of animals, and that in poultry it applied to the spleen as well as the liver. At that time it had been shown (Brenner, Brookes & Roberts, 1942) that female rats could store vitamin A in their livers with much greater facility than males, and it was suggested by Widdowson & McCance that the storage of vitamin A might in some way be coupled with that of iron. More recently, Moore & Sharman (1950) and Moore, Sharman & Ward (1951) have pointed out that the level of dosage makes a great difference to the ease with which vitamin A is accumulated in the organs of the rat. At comparatively low intakes (40 i.u./day) the liver of the female contained over twice as

much vitamin A/g. as that of the male, whereas the kidney of the male contained over 5 times as much as that of the female. At higher levels of dosage (640–1280 i.u./day) these differences tended to disappear. This work suggested that varying the dose might affect storage of iron by the organs of male and female rats in a somewhat similar way, and drew attention to the fact that information about sexual differences in the storage of iron by the rat had so far been confined to the liver. The work now to be described was accordingly undertaken.

EXPERIMENTAL

Black and white hooded rats were employed for these experiments. The litters from sixteen females, which had been mated at the same time, were used. The litters were all born within 11 days. Forty-five males and forty-three females, two or three of each sex from each litter, were used for analysis at birth, and the number in all litters was then reduced to nine. At the end of 15 days, twelve males and twelve females, representing all sixteen litters, were taken for analysis. The remaining rats were weaned at 21 days. The oldest were maintained on the stock diet for 11 days until the youngest had been weaned. The average age at the end of this time was 28 days. Then the rats were paired according to age, weight and litter, so that there were two groups of males and two groups of females as similar as possible in all these respects. All were given unlimited amounts of the stock diet, consisting of a mixture of bread, whole wheat, oat flakes, bran, wheat germ, yeast, fishmeal and dried milk, with liver once a week and greens or carrots every alternate day. They were also given fresh whole milk every day. The stock diet contained approximately 3 mg. Fe/100 g. fresh weight. At the beginning of the experiment, when the rats were growing rapidly, they ate more food in proportion to their body weight than when they were older, and their food provided them with about 2·5 mg. Fe/100 g. body wt./day. After the experiment had been running for 3 months and the rats were nearly fully grown the food supplied only 0·5 mg. Fe/100 g. body wt./day.

One group of each sex received additional iron as $FeSO_4$. This was made up as 15% (w/v) aqueous $FeSO_4 \cdot 7H_2O$ containing 30 mg. Fe/ml. The supply for each day was measured with a pipette and added to the fresh milk. The amount of additional iron given was equivalent to 3 mg. Fe/100 g. body wt./day throughout the experiment. The rats readily drank the milk containing the iron. A record was kept of any left over, but this was insignificant in amount. The rats were weighed weekly throughout the experiments. Twenty-two days after the administration of iron had begun, when the average age of the rats was 50 days, four males and four females were taken from each group. A similar number of rats was taken at the age of 100 and 150 days, and three of each sex were taken at 200 and 250 days. The rats were killed by a blow on the head. The livers, spleens and kidneys were removed, washed rapidly with distilled water, blotted on filter paper and weighed. The organs from all the rats in each group were pooled and homogenized with 3–50 times their weight of water. Samples of the homogenate were measured out for the determination of inorganic iron. This was estimated with 2:2′-dipyridyl (Shackleton & McCance, 1936). The solid matter was removed by centrifuging, and the intensity of the red colour was matched in a Spekker absorptiometer, using an Ilford green filter no. 604 (Harrison, 1952).

RESULTS

Table 1 shows the average weights of the animals at seven stages of growth, and the weights of the organs as percentages of the body weight. The rate of growth and the weights of the organs did not appear to be affected by varying the dosage of iron.

Fig. 1 shows the concentration of inorganic iron in the liver, spleen and kidney of the male and female rats at the normal (lower) levels of iron intake. The

Table 1. *Average weights of the bodies and organs of the male and female rats used for these experiments*

(Weights of organs are expressed as percentage of body weight.)

Age (days)	No. of animals in each group	Body wt. (g.)		Liver (as % body wt.)		Spleen (as % body wt.)		Kidneys (2) (as % body wt.)	
		Control	+Fe	Control	+Fe	Control	+Fe	Control	+Fe
				Males					
0	45	5·29	—	5·9	—	0·23	—	1·46	—
15	12	24·3	—	4·7	—	0·34	—	1·46	—
50	4	154	148	5·2	4·6	0·41	0·61	0·97	0·89
100	4	278	278	3·1	3·3	0·26	0·35	0·69	0·73
150	4	300	297	3·2	2·9	0·24	0·25	0·72	0·67
200	3	332	330	3·4	3·2	0·25	0·23	0·68	0·62
250	3	338	367	2·8	2·8	0·19	0·24	0·65	0·69
				Females					
0	43	4·79	—	7·3	—	0·19	—	1·40	—
15	12	25·5	—	5·5	—	0·31	—	1·39	—
50	4	116	111	4·5	4·7	0·28	0·38	0·95	0·96
100	4	193	194	3·3	3·3	0·40	0·28	0·75	0·75
150	4	225	228	3·4	3·0	0·33	0·21	0·75	0·68
200	3	228	227	3·0	3·1	0·28	0·27	0·70	0·67
250	3	248	244	3·0	3·1	0·27	0·24	0·67	0·70

initial values were similar in all the organs, and the fall during the suckling period, already well known

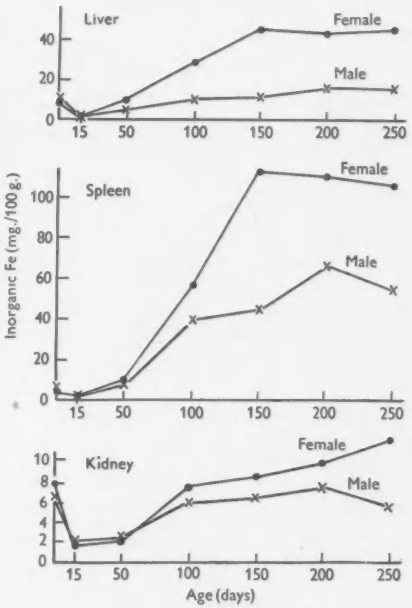

Fig. 1 The effect of sex on the concentration of inorganic iron in the organs of rats.

to take place in the liver, clearly affected also the kidney and spleen. Thereafter the concentration of iron increased in all three organs, but to very variable extents, and the percentages found in the female always exceeded those in the male tissues.

Table 2 shows the results of administering additional iron. Since the iron supplement only commenced after weaning, the earliest age at which any effects could be expected was 50 days, but by this time the additional iron given in the food had raised the concentration in the liver, spleen and kidney of both the male and female rats, and the differences increased with the age of the rats, although the results were somewhat variable. Increasing the intake of iron did not, however, at any age cause the concentration of iron in the male liver and spleen to exceed that in the female organs.

DISCUSSION

The effect of sex on the concentration of inorganic iron in the organs of the rat is evidently not confined to the liver. The kidney and spleen, and possibly other organs, are also involved. There are two ways in which this might be brought about: (1) since, as is now generally agreed, the amount of iron in the body is regulated by the rate of absorption rather than the rate of excretion (McCance & Widdowson, 1943, 1944), the effect of sex may be confined to the epithelium of the gut, or even to the pH or to some physicochemical equilibrium within the lumen. (2) The quantity of ferritin, or other iron-binding proteins, or both, may be much greater in the organs of the female than in those of the male, and consequently the equilibrium in the absorption-circulation-storage system may be more easily maintained in favour of the first. These present results provide no evidence for either hypothesis. They do, however, suggest that the storage of iron

Table 2. *Effect of the administration of additional iron on its concentration in the organs of male and female rats*

	Inorganic Fe (mg./100 g. organ, fresh wt.)					
	Liver		Spleen		Kidneys	
Age (days)	Control	+Fe	Control	+Fe	Control	+Fe
			Male rats			
0	10·7	—	6·1	—	6·6	—
15	1·6	—	0·8	—	2·0	—
50	4·5	8·2	7·2	12·9	2·3	3·2
100	9·7	21·4	39·7	68·0	5·7	10·8
150	10·8	15·6	44·2	52·7	6·1	8·8
200	15·2	28·0	66·1	110·0	7·2	10·7
250	15·2	27·3	54·0	102·0	5·2	8·3
			Female rats			
0	8·1	—	4·5	—	7·8	—
15	1·6	—	1·5	—	1·7	—
50	10·0	18·5	10·0	16·0	2·1	3·1
100	28·6	50·2	56·3	103·0	7·3	Lost
150	44·5	81·7	112·0	160·0	8·3	10·3
200	42·2	60·0	110·0	118·0	9·6	11·0
250	43·3	49·8	106·0	138·0	11·9	14·9

and vitamin A are quite independent processes, for with the limited dosages of iron which have been employed the organs of the female have always contained higher concentrations of iron, and there has been no suggestion of a reversal of the female-male relationship. The vitamin A intake in this investigation was about 100 i.u./rat/day, and at this level the amount in the kidneys of the males probably exceeded very materially that in the corresponding organs of the females.

SUMMARY

1. Higher concentrations of iron were found in the livers, spleens and kidneys of female rats than in the corresponding organs of male rats.

2. The differences were greatest at and after the 100th day of life.

3. Increasing the intake of iron 2–6 times raised the concentrations found in all three organs, but did not alter the female-male relationship.

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